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ENDOCRINOLOGY

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THE EFFECT OF ADRENAL CORTICAL HORMONES ON THE INCORPORATION OF C^{14} INTO THE PROTEIN OF LYMPHATIC CELLS*

SAUL KIT† AND E. S. GUZMAN BARRON

From the Chemical Division, Department of Medicine, The University of Chicago, Chicago, Illinois

DURING the last years there have appeared a large number of publications on the effect of adrenal hormones on metabolic reactions studied either *in vivo* or *in vitro*. The influence of the adrenal cortex upon nitrogen metabolism is well known (see reviews by Sayers, 1950, and by Ingle, 1950). The numerous attempts to determine the mechanism of this action, however, have been unsuccessful. Some authors have reported that the activity of liver amino acid oxidase and of proline oxidase in kidney is diminished after adrenalectomy and is brought back to normal on injection of corticoid hormones (Cagan *et al.*, 1950; Jensen and Gray, 1951; White *et al.*, 1948); while others found that D-amino acid oxidase in kidney is inhibited by desoxycorticosterone (Hayano *et al.*, 1950). It has also been reported that the synthesis of chondroitin sulfate (Layton, 1951a) and of mucopolysaccharide (Layton 1951b) in the rat is inhibited by cortisone. In the metabolism of carbohydrates the antagonistic influence of insulin and corticoid hormones is accepted, although the mechanism of this action is unknown. It has been reported that glycogen formation in muscle is decreased in adrenalectomized rats (Mentha *et al.*, 1948) and that desoxycorticosterone inhibits glycogen production and breakdown in both normal and adrenalectomized rats (Boyonic *et al.*, 1949). Bartlett and MacKay (1949) found that desoxycorticosterone reduced glycogen formation in skeletal muscle. The effect of this hormone on the glycolysis of skeletal muscle depends, according to Dirscherl and

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† Postdoctoral Fellow, U. S. Public Health Service.

Brener (1951), on the rate of glycolysis of the tissue. The same antagonism between insulin and corticoid hormones seems to exist in their influence on fat metabolism. Pretreatment with cortisone diminished the incorporation of C^{14} into the fatty acids of the mammary gland of lactating rats, whereas insulin increased it (Balmain *et al.*, 1952). Experiments on tissue respiration have shown only inhibitions (Hayano *et al.*, 1950; Gordan *et al.*, 1951).

It is difficult to conceive how the steroid hormones would have such varied activities unless it is postulated that their action is indirect, that they act as a regulatory agent of cellular metabolism. It might be impossible to discover such a function in experiments *in vivo* because of the functional interrelationships of the hormones, and in experiments *in vitro* with isolated enzyme systems or homogenates because interphases and the role of the cellular membrane are disregarded. For this reason, it was decided to examine the problem at the level of the intact cell. The marked sensitivity of lymphatic tissue to the action of adrenal cortical secretions (Daugherty and White, 1945; Molomut *et al.*, 1950) prompted us to choose these tissues as the experimental material. We present in this paper experiments on the effect of some of these hormones on the incorporation of C^{14} into the proteins of lymphatic cells and on the rate of oxidation of some carbohydrate intermediates, as well as on glycolysis.

EXPERIMENTAL

The lymphatic cell suspensions were obtained from the following tissues: the appendix of the rabbit, thymus of rats, spleen of rats and mice, and Gardner lymphosarcoma from C3H mice. Cell suspensions were prepared by grinding the tissues gently in a previously cooled aluminum cylinder, one end of which contained a Monel metal screen (60 mesh). The cells were washed through the screen with ice-cold Ringer-phosphate. In a room at 3° C. they were centrifuged for five minutes at 2,000 r.p.m. The packed cells were diluted with about twice their volume of ice-cold Ringer-phosphate, and an aliquot was taken—usually 0.5 ml. per vessel. The hormones, dissolved in methanol, were placed either in glass-stoppered flasks or in Warburg vessels. The methanol was removed by evacuation leaving a thin film of dry hormone at the bottom of the vessel. The cell suspensions with the hormone were kept in the 3° C. room for one hour to facilitate penetration or adsorption of the hormone; it was found later that the preincubation period had no effect on the influence of the hormone. Glucose, labeled glycine ($CH_2NH_2C^{14}OOH$), labeled acetate ($CH_3C^{14}OONa$) or labeled phenylalanine ($C_6H_5C^{14}H_2CHNH_2COOH$) was added just before incubation of the flasks or Warburg vessels at 38° C. in Warburg baths shaken

at a rate of 100 cycles per minute. Protein precipitation and preparation of the samples for radioactivity assay were performed as described by Farber *et al.* (1951). Counts were made with a Radiation Counter Laboratories three position gas flow counter. An empirically determined curve was used for absorption corrections and samples were counted for a sufficient length of time to reduce the counting error to 3%. The radioactive glycine and acetate were prepared at the laboratory; radioactive phenylalanine was acquired from Tracerlab; 17-hydroxy-11-dehydrocorticosterone

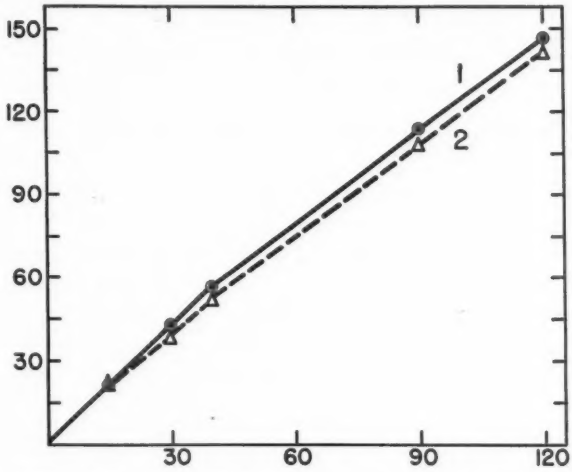

FIG. 1. The Effect of Compound F (100 micrograms) on the Respiration of Rat Thymus. Buffer, Ringer-phosphate, pH 7.4 Substrate. 0.01*M* glucose, 0.001*M* glycine. Abscissa, time in minutes; ordinate, O_2 uptake in c. mm. 1. Control; 2. Compound F.

acetate (Cortisone) was kindly provided by Merck and Co; 17-hydroxycorticosterone (Compound F) was a gift from Drs. R. Landau and C. Huggins of this University.

Effect of Compound F on the Respiration and Glycolysis of Lymphatic Cells.—It has been observed by others (Hayano *et al.*, 1950; Gordan *et al.*, 1951) that high concentrations of several steroid hormones inhibit the respiration of animal tissue slices and homogenates. This was also found to be true in the case of lymphatic tissue when 200 micrograms per ml. or more of Compound F, desoxycorticosterone, or testosterone were added to flasks containing the equivalent of 20 mg. dry weight of tissue. At a concentration of 100 micrograms per ml. Compound F. however, had little effect on the respiration of rat thymus cell suspensions in the presence of glucose and of glycine-1-C^{14} (Fig. 1). It had little effect also on the oxidation and the utilization of pyruvate, of α ketoglutarate, and of succinate

TABLE 1. EFFECT OF COMPOUND F (100 MICROGRAMS) ON THE OXIDATION AND UTILIZATION OF SOME INTERMEDIATES OF THE CITRIC ACID CYCLE

Cell suspension, 1 ml. Pyruvate, 0.005*M*; α ketoglutarate, 0.005*M*; succinate, 0.02*M*. Buffer, Ringer-phosphate. Temp. 38° C. Incubation 2 hrs. Appendix from rabbit, and spleen from mouse. Experiments marked with asterisk are from rat spleen. Figures in table are means of duplicates.

Substrate	Measurement	Appendix		Spleen	
		Control	Hormone	Control	Hormone
None	O_2 uptake, cmm.	215	210	228	192
Pyruvate	O_2 uptake, cmm.	272	253	240	213
Pyruvate	Utilization,[1] cmm.	3.4	3.4	2.0	1.9
α Ketoglutarate	O_2 uptake, cmm.	—	—	247	213
α Ketoglutarate	Utilization,[1] cmm.	—	—	0.4	0.4
None		—	—	138*	111*
Succinate		—	—	172*	141*

[1] Microliters substrate equivalent per mg. dry weight per hour.

by the lymphatic cells from rabbit appendix, and from rat and mouse spleen (Table 1). In the large number of experiments performed the inhibition of respiration in appendix cell suspensions reached a maximum of 7%; in thymus a maximum of 13%; and in spleen a maximum of 19%. The respiration of lymphosarcoma cells was not affected.

The anaerobic glycolysis of appendix lymphatic cells was inhibited 10% with 50 micrograms of Compound F, and 27% with 100 micrograms (Fig. 2).

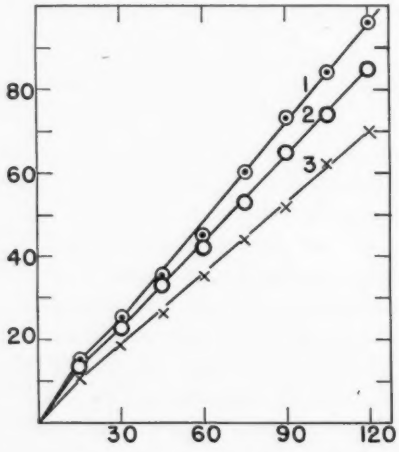

FIG. 2. Effect of Compound F on the anaerobic glycolysis of the lymphatic cells of rabbit's appendix. Buffer, Ringer-bicarbonate saturated with N_2:CO_2; pH 7.4. Glucose, 0.01*M*. Temp. 38° C. Abscissa, time in minutes. Ordinate, CO_2 production in c.mm. Dry weight of cells, 2.3 mgs. 1. Control; 2. 50 micrograms of Compound F; 3. 100 micrograms

Effect of Some Steroid Hormones on the Incorporation of C^{14} into the Protein of Lymphatic Cell Suspensions.—A number of preliminary notes have appeared reporting the influence of adrenalectomy and of adrenal cortical hormones on the metabolism of isotopic glycine, such as increased urinary nitrogen on administration of cortisone to adrenalectomized rats (Clark, 1950) increased incorporation of N^{15} glycine into the tissue proteins of adrenalectomized mice (White *et al.*, 1948) diminished incorporation of alanine-1-C^{14} by rat liver slices and diaphragm on addition of cortisone

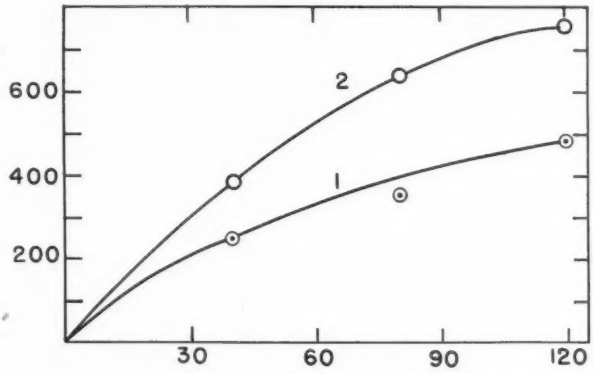

FIG. 3. The Incorporation of C^{14} into the Proteins of Rat Thymus at Different Intervals after Addition of Glycine-1-C^{14}. Abscissa, time in minutes. Ordinate, counts per min. per mg. protein. 1. In the presence of 100 micrograms Compound F; 2. Control.

(Sinex, 1951). When rat thymus cells were incubated with glycine-1-C^{14}, the incorporation of C^{14} into protein was steadily inhibited on addition of 100 micrograms of Compound F per ml. (Fig. 3). This inhibition was observed in the absence of any inhibition of respiration (Figure 1). Furthermore, while the degree of inhibition of C^{14} incorporation into appendix cell protein increased with the concentration of Compound F added to the cell suspensions, the small inhibition of respiration (around 10%) remained unaltered (Fig. 4). In the presence of glycine-1-C^{14} the incorporation of C^{14} into protein by rabbit appendix was inhibited 23% on addition of 100 micrograms of Compound F; the inhibition in rat thymus was 37%; in rat spleen 19%. Compound E (100 micrograms) inhibited 18% the incorporation of C^{14} into the proteins of rat spleen; 200 micrograms inhibited 23% in rat thymus. Neither Compound F nor Compound E (100 micrograms) had any significant effect on the incorporation of C^{14} into the proteins of lymphosarcoma cells. The same lack of effect was found on addition of 200 micrograms of Compound E, but 200 micrograms of Compound F brought about 17% inhibition (Table 2). An increase in the glycine concentration

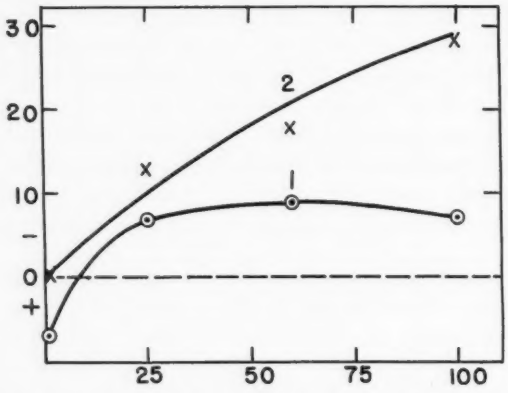

FIG. 4. Effect of Compound F on the Respiration and C^{14} Incorporation into the Protein of Appendix (rabbit) Lymphatic Cells. Buffer, Ringer-phosphate. Substrates, 0.01*M* glucose and 0.001*M* glycine-1-C^{14} (81,000 cm). Ordinate, per cent increase (+) or decrease (−); Abscissa, Compound F in micrograms; 1. Respiration; 2. C^{14} incorporation into protein.

from 1 mM per flask to 2 mM had no effect on the incorporation of C^{14} or on the degree of inhibition by the hormone. The uptake of glycine by mouse spleen or by lymphosarcoma cells is increased in the presence of glucose (Kit and Greenberg, 1951). The same increase was observed in rabbit appendix cells and in rat thymus cells. However, the omission of glucose did not increase the inhibition of C^{14} incorporation into protein produced by Compound F. Cholesterol, at concentrations as high as 500 μg. per 1 ml. did not affect glycine uptake.

TABLE 2. EFFECT OF STEROID HORMONES ON THE INCORPORATION OF GLYCINE 1-C^{14} INTO THE PROTEIN OF LYMPHATIC CELL SUSPENSIONS

Buffer, Ringer-phosphate containing 0.01*M* glucose and 0.001*M* glycine 1-C^{14} (81,000 c./m. per flask). Dry weight of cells, about 20 mgs. Incubation, 2 hrs. Temp. 38°. Gas phase, air. The figures in the table are the mean of triplicate determinations ± standard error of the mean. Hormone, 100 micrograms per vessel, except those indicated with asterisk where it was 200 micrograms. Each planchet contained about eight mg. of protein.

Tissue	C^{14} in protein (counts/min./mg. protein)			
	Compound F		Compound E acetate	
	Control	F	Control	F
Rabbit appendix	417 ±16	319 ±31		
Rat thymus	830 ±40	586 ±26	900* ±29	689* ±29
Rat thymus	919* ±44	477* ±50	—	—
Rat thymus without preincubation with hormone	923 ±81	621 ±27	—	—
Rat spleen	49.0 ± 1.4	35.4 ± 5.1	247 ±23	202 ±13
Mouse spleen	273 ±14	220 ±12	—	—
Lymphosarcoma (mouse)	208 ±10	199 ±13.8	192 ± 5.5	180.5* ± 8.5
	162 ± 5.2	134* ± 3.2		

TABLE 3. EFFECT OF COMPOUND F ON THE INCORPORATION OF DL-PHENYLALANINE-3-C^{14} INTO THE PROTEIN OF THE LYMPHATIC CELLS OF RABBIT'S APPENDIX

Buffer, Ringer-phosphate, pH 7.4. Glucose, 0.01*M*. D-L-phenylalanine, 0.002*M* (427,600 c./m.). Dry weight of cells, 21.1 mgs. Incubation time, 2 hours. Compound F, 100 micrograms. Figures are mean of triplicate determinations. Each planchet contained about 8 mg. protein.

Exp. measurements	Control	Hormone
O_2 uptake, c.mm.	225	210
C^{14} in protein, c./min./mg. Protein	1307 ± 12	1005 ± 35

Beta labelled phenylalanine is rapidly incorporated into the protein of appendix cells. Compound F (100 micrograms) inhibited this process 23% (Table 3) although respiration was unaffected.

The labelling of the protein was in this case attributable to phenylalanine alone. The protein was hydrolyzed with HCl and the amino acids separated by paper chromatography, using butanol-acetic acid-water (100:22.5:50) as the solvent. Radioactivity was observed only in the area corresponding to phenylalanine; none was observed in the tyrosine or dicarboxylic amino acid spots. Absence of C^{14} in the respiratory CO_2 had previously been observed after beta labelled phenylalanine was incubated with lymphosarcoma cells (Kit and Greenberg, 1951).

In another series of experiments, acetate-1-C^{14} was used as the C^{14} donor. In this case C^{14} enters the protein molecule mainly as glutamic and aspartic acids through oxidation of acetate *via* the tricarboxylic acid cycle (1). The O_2 uptake and the respiratory $C^{14}O_2$ of appendix and lymphosarcoma cells were not affected at all by 100 micrograms of Compound F; C^{14} incorporation into the proteins of appendix cells was 19% inhibited; of thymus cells, 28%. Incorporation of C^{14} into lymphosarcoma cells was not affected (Table 4). Addition of $6 \times 10^{-4} M$ of taurocholate to appendix cells had no effect on the C^{14} incorporation into their proteins.

TABLE 4. EFFECT OF COMPOUND F (100 MICROGRAMS) ON THE RESPIRATION OF APPENDIX, THYMUS, AND LYMPHOSARCOMA CELLS, AND ON THE INCORPORATION OF C^{14} FROM ACETATE 1-C^{14} INTO PROTEIN

Lymphosarcoma cells washed four times with K-Ringer phosphate. $CH^3C^{14}OONa$, 0.01*M* (326,000 c./m. per flask). Cell volume, 0.5 ml.; K-Ringer-phosphate 0.4 ml.; acetate, 0.1 ml. Incubation time, 120 minutes for normal cells, 150 minutes for lymphosarcoma cells. Each planchet contained about 8 mg. of protein.

Exp. Conditions	Appendix		Lymphosarcoma		Thymus	
	Control	Hormone	Control	Hormone	Control	Hormone
O_2 Uptake, c.mm.	220	218	289	289	—	—
Resp. $C^{14}O_2$, Total c./m. in $BaCO_3$	16,940	17,571	5,651	4,708	—	—
C^{14} in Protein c./m./mg. Protein	302	242	17.6	16.0	732	523

DISCUSSION

Evidence is accumulating in favor of the view that the adrenal glands secrete mainly Compound F and that many of the 28 steroids isolated by chemical methods are devoid of physiological importance. For this reason these studies have dealt primarily with the effects produced by Compound F. From the evidence presented in this paper it may be concluded that whereas Compound F, in relatively small concentrations ($3\times10^{-4}M$) had a small inhibitory effect on the respiration of lymphatic cells, it definitely inhibited the incorporation of radioactive amino acids into the protein of these cells. This inhibition may be interpreted as an inhibition of protein synthesis by a mechanism still unknown. It is unlikely that the results can be attributed to increased protein catabolism due to the action of the hormone, although a more rapid protein breakdown would have diluted the labeled glycine with inert glycine ant thus have induced an apparent inhibition of uptake. This follows from the fact that the degree of inhibition was not changed on doubling the C^{14}-glycine concentration. Also, unpublished data from this laboratory indicate that the amount of labeled glycine added to each flask was several times the amount of the endogenous glycine.

Less than one per cent of the added glycine was oxidized to CO_2 in these experiments. Addition of hormone produced no increase in the rate of oxidation.

The apparently greater resistance of the lymphosarcoma cells to the inhibitory action of Compound F on protein synthesis is of interest and perhaps has some relation to the autonomy of the cancer cells. The regulation of protein synthesis by the adrenal hormones might have been lost in the tumor cells.

SUMMARY

The respiration of lymphatic cells obtained from rabbit appendix, rat and mouse spleen, rat thymus, and from the Gardner mouse lymphosarcoma was slightly inhibited by 17-hydroxycorticosterone and by 17-hydroxy-11-dehydrocorticosterone. Oxidation of some of the intermediates of the tricarboxylic acid cycle was also little affected, whereas there was definite inhibition of the anaerobic glycolysis of appendix lymphatic cells. The C^{14} incorporation into the protein of the normal lymphatic cells on addition of C^{14} labeled glycine, acetate, and DL-phenylalanine was inhibited by the adrenal hormone. Incorporation into the proteins of lymphosarcoma cells was not affected by hormone addition similar to that used in normal cells, a higher concentration of hormone being required to obtain inhibition.

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STUDIES ON THE AMINO ACID CONTENT OF PREPARATIONS OF ANTIDIURETIC SUBSTANCE OBTAINED FROM PATHOLOGICAL URINE AND FROM COMMERCIAL PITUITRIN

CLAUDIO MALAGUZZI VALERI, M.D., MICHELE ZACCO, M.D.,* AND MARZIO PERRINI, M.D.

From the Institute of Internal Medicine of the University of Bari, Italy

THIS paper reports the results of the application of two dimensional paper chromatography to the identification of the amino acid composition of antidiuretic material prepared both from commercial pituitrin and from urine colected in the premenstruum of women affected by the premenstrual syndrome. Further work on antidiuretic substance prepared from cirrhotic urine is being completed and will be reported in another paper.

Symptoms of water retention are the most characteristic for cases of "premenstrual syndrome" (Heilig, 1924). This would explain the periodical increase of body weight in these cases (Okey and Stewart, 1933). In some patients this phenomenon is so marked that it results in localized and/or generalized edema (Thomas, 1933; Molnar and Gruber, 1934; Sweeney, 1934, Atkinson and Ivy, 1936; Thorn *et al.*, 1938). This periodic edema can assume great clinical importance. It has been observed, for example, that women with mitral stenosis may develop recurrently with menses acute pulmonary edema (Edeiken and Griffith, 1940; Lenègre and Coblentz, 1947; Chini, 1949). Hyperfolliculinemia has been suggested as the cause of these phenomena (Lenègre *et al.*, 1947); Lutembacher and Gallimard, 1948; Schwob *et al.* 1948; Chini, 1949).

High urinary antidiuretic titer in association with clinical edema has been observed in the premenstruum (Robinson and Farr, 1940; Edeiken and Griffith, 1940; Lloyd and Lobotsky, 1949, 1950). Administration of estrogens results in a measurable increase in antidiuretic substance in the urine of certain women (Shapiro, 1938; Gilbert-Dreyfus *et al.* 1950; Malaguzzi-Valeri, and Zacco, 1951). Posterior hypophysial hyperfunction has been indicated as a possible cause of the antidiuretic substance in blood and urine (Anselmino and Hoffmann, 1931, 1936; Cushing, 1934).

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* At present U. S. Public Health Service Fellow, Department of Research Medicine, University of Pennsylvania.

The object of this paper is to point out the striking similarity between the amino acid content of the antidiuretic principle isolated from three different commercial pituitrin preparations and the antidiuretic principle extracted from the urine of 5 cases of "premenstrual syndrome."

EXPERIMENTAL

Starting Materials.—The antidiuretic factor of pituitrin from three different commercial preparations was isolated and purified using the method of ascending paper chromatography (Filter paper Whatman No. 1). Phenol

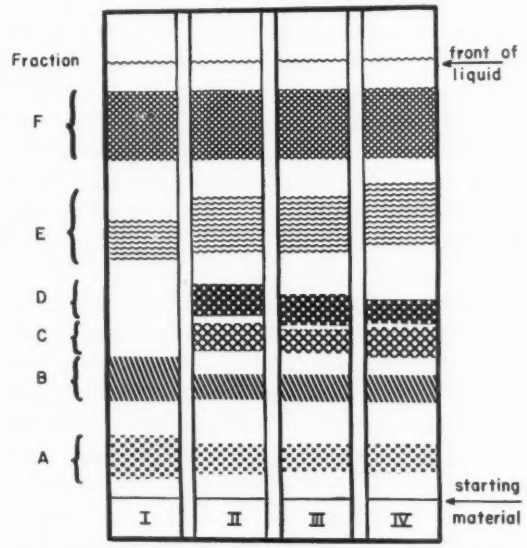


FIG. 1. Chromatograms of twenty units of commercial posterior pituitary extract. I. Refisal; II. Postipophisan; III. Piton; IV. Chromatogram of an aliquot of the pathological urinary extract corresponding, for its activity in the rat, to about twenty units of commercial posterior pituitary extract.

saturated with water plus 0.8% of ammonia was used as solvent. One of the commercial preparations (Fig. 1, I) showed on chromatographic separation 4 different components. The other two preparations (Fig. 1, II and III) showed 6 different components. Ninhydrin was used as the developing agent. Only the two fastest components of each commercial product, which we call fraction E and fraction F (Fig. 1), demonstrated antidiuretic activity in rats. The Rf values of the active fractions were comparable to each other regardless of the origin of the product. The antidiuretic titer of every fraction was tested according to Gilman and Goodman (1937) by

injecting the material subcutaneously in rats previously hydrated with water *per os*, and by determining the rate of diuresis in the subsequent two hours. The percentage of urine excreted was referred to the water load. The antidiuretic activity per unit weight of fraction F when compared to the whole original commercial preparation showed a 15 to 17 fold increase. Fraction E had only 75% activity of the corresponding fraction F. Other fractions (A,B,C,D) were inactive. Twenty oxytocic units of commercial pituitrin were used for the chromatographic procedure. Each fraction (from A to F) was eluted separately and tested in individual rats. The inhibition of diuresis compared to the controls was from 90 to 100 per cent when using fractions F and from 70 to 80 per cent when using fractions E. Twenty oxytocic units of the original commercial preparation corresponded to from 770 to 800 μg. of protein material, which is approximately one unit per 40 μg. of protein material. Purified fractions E and F, originally from some twenty units of commercial pituitrin, showed increased potency corresponding to 3.4 and 2.3 μg. respectively, per unit of standard power.

Hydrolysis.—The two active chromatographic fractions, namely E and F, originally forming a pool of 200 to 300 units of commercial pituitrin, were each eluted from the paper with a 0.25% acetic acid solution and lyophilized. The dried material was dissolved in glacial acetic acid and precipitated with 9 to 10 volumes of a mixture of equal parts of ether and petroleum ether. After centrifuging the precipitated material was rapidly dried. An amount of material corresponding to 20 to 30 units of pituitrin was then rechromatographed and its distribution and biological activity were checked again. The remaining dried precipitate was hydrolyzed, as recommended by Stein and Moore (1948) for 20 hours in 200 volumes of 5.8 N HCl which had been distilled twice in glass. There was no visible humin precipitate. The solution, however, was yellow following hydrolysis. The sample was then evaporated to dryness from four to six times *in vacuo*, and made up to a volume of 2 ml. with water. For ascending chromatographic analysis aliquots of this solution were delivered directly on the paper and dried by using a fan. Phenol saturated with water plus 0.8% ammonia was used as first solvent. A beaker was introduced into the cabinet containing water saturated with phenol, 3% ammonia and approximately 0.2 gm. KCN. After 60 to 72 hours the paper was dried at room temperature in front of an electric fan. Thereafter the sheet was appropriately set up for a two dimensional chromatogram using n-butanol-acetic acid and water as the second solvent (Partridge, 1948).

The antidiuretic substance of urine collected in the premenstruum in cases of premenstrual syndrome was prepared by using a modification of the method of Noble (1938). The urine, which had been collected during a period of 24 to 48 hours, was evaporated to one tenth of the initial volume.

Acetone was then added until no further precipitate formed. The precipitate, recovered by filtration, was dried in air, redissolved in glacial acetic acid and filtered. 9 to 10 volumes of a mixture of ether and petroleum ether were then added to this filtrate in a separatory funnel. Immediately a viscous, blackish and rapidly sedimenting precipitate was obtained. This was discarded. Finally, a light brown, slowly settling material appeared. This material was recovered by filtration, dried and redissolved in 2 to 3 ml. of a 0.25% solution of acetic acid. This solution possessed antidiuretic activity and was studied by the method of paper chromatography. The chromatograms obtained from the urine preparations of a number of patients were similar to the chromatograms obtained from the commercial pituitrin. Six substances (A,B,C,D,E,F) (Fig. 1, IV) appeared on the chromatogram of the urine samples. Position, size, and ninhydrin colors of these urinary substances gave a chromatographic picture similar to that obtained on fractionation of pituitrin (Fig. 1). Again only the two fastest fractions, E and F, showed antidiuretic activity in the rat. For the chromatographic procedure an amount of urinary antidiuretic substance was used that corresponded, for its activity in the rat, to about 20 units of commercial pituitrin.

The precipitate obtained from specimens of urine collected in the intermenstrum of the same patients and in the premenstruum of normal women up to 72 hours, did not show the usual chromatographic pattern and did not give any evidence of antidiuretic activity.

The two active urinary fractions, i.e. E and F, after elution from the paper, were separately lyophilized, dissolved with glacial acetic acid, precipitated with ether and petroleum ether, and dried. The chromatographic distribution and the biological activity of an aliquot of these two fractions were checked again. The remaining dried material of each fraction was hydrolyzed and chromatographed following the same procedure as for the pituitrin fractions E and F.

AMINO ACID ANALYSIS

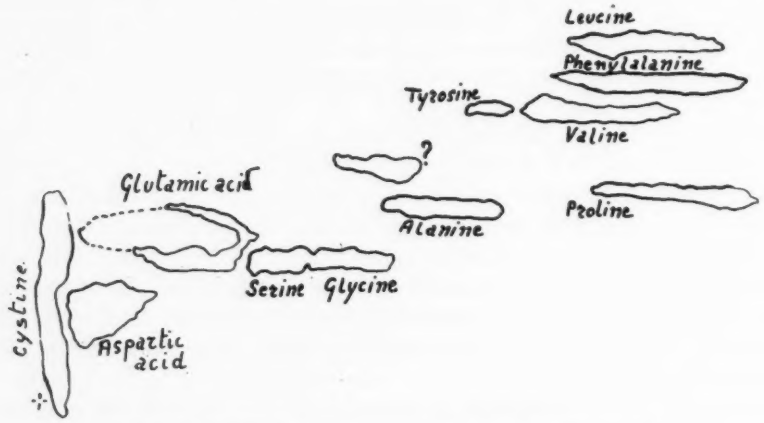
Pure phenol saturated with water plus 0.8% of ammonia was always used as the first solvent for ascending chromatograms (room temperature). Just before closing the cabinet at the beginning of a run an beaker was introduced containing water saturated with phenol plus 3% of ammonia and 0.2 gm. of KCN, the latter to prevent oxidation of phenol. After about 60 to 72 hours the sheets were dried at room temperature by an electric fan.

After phenol, two-dimensional chromatograms were obtained by using n-butanol-water-acetic acid (Partridge, 1948) as second solvent.

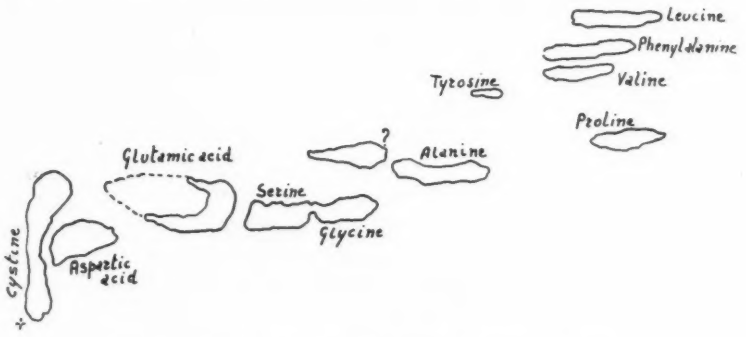
Standard solutions of pure amino acids made up in 75% (by volume)

MAP. 1. Artificial amino-acid mixture.

ethanol were used delivering 15 to 25 gamma of each from a calibrated pipette directly on the sheet in a small spot at the starting point. The optical species has not been indicated, since there is evidence that the active compounds and racemic mixtures travel similarly on the paper (Dent, 1948). The maps of the spots are drawn to scale. Rf values are not mentioned as such since it is commonly agreed that the relative position of spots is a better guide to identity. Under our conditions the pattern remained remarkably constant and therefore was used exclusively as a method of identification. In a number of chromatograms marker amino acids were added to the unknown amino acid mixture obtained by



MAP 2. Hydrolysate from commercial pituitrin, Fraction F.

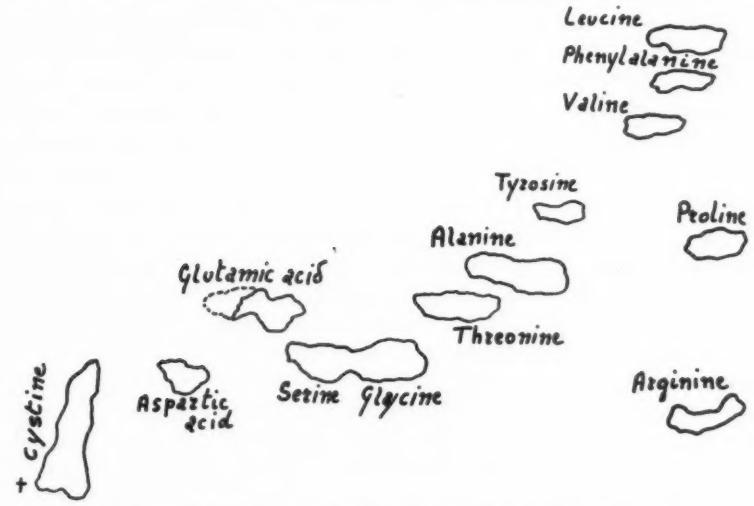


MAP 3. Hydrolysate from pathological urine, Fraction F.

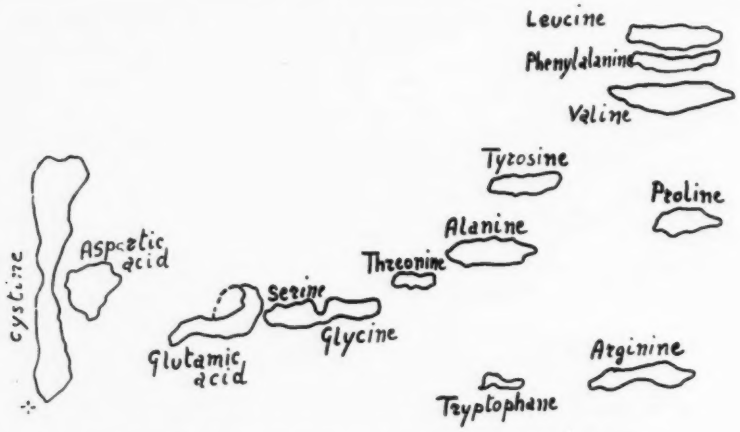
hydrolysis of the active fractions. The color developed by the ninhydrin reaction gave additional clues to the identity of some spots (Maps 1, 2, 3, 4, and 5).

RESULTS

As shown in the maps the two fastest and most active fractions F from both pituitrin and urine, yield on hydrolysis 11 amino acids, viz., cystine, aspartic acid, glutamic acid, serine, glycine, alanine, tyrosine, proline, valine, phenyalanine, leucine, and one unknown spot. The two relatively slower and less active fractions E both from pituitrin and urine show on hydrolysis 13 amino acids, i.e., the same 11 amino acids found in the hy-



MAP 4. Hydrolysate from commercial pituitrin, Fraction E.



MAP 5. Hydrolysate from pathological urine, Fraction E.

drolysate of fractions F, plus threonine and arginine. The unknown spot is not found in fraction E. In the hydrolysate of fraction E from urine one more spot has been occasionally identified. This spot was not constant in all of our preparations. On the map it occupies the position of tryptophane. It is questionable, however, whether one would expect to see tryptophane after acid hydrolysis.

The abnormal shape and non-homogeneous ninhydrine color of some spots, e.g., cystine and glutamic acid, call for some comment. The large spot which occupies the position of cystine in our maps may be due to a mixture of cystine and partial oxidation products which can appear during hydrolysis or during chromatography in the alkaline atmosphere produced by the ammonia vapor (Joslyn, and Stepka, 1949). Glutamic acid is known to show non-homogeneous ninhydrin color reaction, the bulk of the color sometimes being collected at the two ends. The lower part is always much redder in color. At other times it may take up an even lower position on the map (Dent, 1948). No reason can be suggested for these unpredictable anomalies of behavior. Together with other anomalies in the behavior of glutamic acid already reported in the literature, these findings suggest that some fundamental facts in the chemistry of this compound still are unknown (Dent, 1948).

DISCUSSION

This paper reports the results of the application of two dimensional paper chromatography to the identification of the amino acid composition of the antidiuretic principle of commercial pituitrin and of the antidiuretic material prepared from urine collected in the premenstruum of five cases

of premenstrual syndrome. The urinary antidiuretic material was precipitated with acetone, recovered with glacial acetic acid, reprecipitated with a mixture of ether and petroleum ether. Both the commercial pituitrin and the urinary material were then chromatographed. These chromatograms are very similar to each other. Of the six substances obtained by the chromatographic procedure from both pituitrin and urinary antidiuretic material only the two fastest fractions showed antidiuretic activity in the rat. The two active fractions of each chromatogram, i.e. E and F, from pituitrin and urinary material, were separately eluted from the paper, lyophilized, redissolved in glacial acetic acid, and precipitated with ether and petroleum ether. Acid hydrolysates of the purified active substances were studied for their amino acid composition by two-dimensional paper chromatography using phenol-water as the first and n-butanol-acetic acid-water as the second solvent. The constant Rf values observed for the two active fractions regardless of the origin of the antidiuretic material and the constant chromatographic pattern obtained from hydrolysates in a large number of experiments were considered a good criterion of purity. The potency of our preparations per unit weight appears to be rather high. The most potent extract reported (van Dyke, 1950) contained a unit in about 2 μg., and the pure or nearly pure oxytocic principle of Pierce and du Vigneaud, (1950) was nearly twice as potent in terms of oxytocic assay.

The identical chromatographic behavior of hydrolysates from purified pituitrin as well as from purified urinary antidiuretic material suggests a common source for both substances. Some current views suggest that the antidiuretic principle may possibly be a fragment of a large protein molecule (van Dyke, 1950; Croxatto *et al.*, 1951). It would be of interest to determine whether the fastest and most active fraction F, showing only 11 amino acid components has also a smaller molecular weight than the slower and less active fraction E, which shows 13 (to 14) amino acid components. The elimination of (tryptophan? and) threonine, and arginine could represent the last step of this process of fragmentation and activation of the original protein molecule.

It is not excluded on the basis of this work that the active principle contains ninhydrin non-reacting components. Also the occurrence of tryptophane in our acid hydrolysates cannot be ruled out. Similarly traces of methionine and isoleucine, which tend to occupy the same chromatographic position of valine and leucine, respectively, might also be present.

SUMMARY

The amino acid composition of purified antidiuretic material from three different commercial preparations and from urine collected in the pre-

menstruum of cases of premenstrual syndrome has been determined by two-dimensional paper chromatography. Two active antidiuretic substances from commercial pituitrin as well as from pathological urine were isolated by a combination of chemical and chromatographic procedures. The most active fraction, from both pituitrin and urine, contained cystine, aspartic acid, glutamic acid, serine, glycine, alanine, tyrosine, proline, valine, phenylalanine, and leucine. The less active substance was shown to contain the same series of amino acids plus threonine, and arginine. The suggestion is made that there may well be a common source for these antidiuretic principles.

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A STUDY OF VARIOUS INFLUENCES ON THE IODIDE CONCENTRATING MECHANISM OF THE RAT THYROID[1]

N. S. HALMI, B. N. SPIRTOS, E. M. BOGDANOVE AND H. J. LIPNER[2]

From the Department of Anatomy, State University of Iowa, Iowa City, Iowa

THE concentration ("trapping") of iodide by the thyroid is one of the most specific functions of this gland and constitutes the first step in thyroid hormone biogenesis. If the subsequent organic binding of iodine is blocked with thiouracil, the thyroid:serum iodide concentration ratio (T/S) can be determined (Vanderlaan and Vanderlaan, 1947). These authors found the T/S of normal rats to be around 25:1. The following findings indicated that the thyroidal iodide trap is profoundly influenced by thyrotrophin (TSH): a) In rats made goitrous by the chronic administration of propylthiouracil (PTU) the T/S rose to an average value of 250:1 (Vanderlaan and Vanderlaan, 1947); b) After hypophysectomy PTU did not have such an effect and the T/S of hypophysectomized, PTU treated rats was found to be around 6:1 (Greer, 1949); c) A single dose of TSH, given to normal rats, caused a transient rise in the T/S (Vanderlaan and Greer, 1950). Greer (1951) observed that a spectacular rise in the T/S occurred in response to PTU in a group of rats with hypothalamic lesions, although their thyroids failed to show morphological changes. He found a similarly responsive iodide trap without any evidence of hyperplasia in the thyroids of hypophysectomized mice grafted with pituitaries and treated with PTU (Greer, 1952). On the basis of these experiments he hypothesized that there are two distinct thyrotrophins: G-thyrotrophin, which regulates the growth of the thyroid, and M-thyrotrophin, which controls the iodide trapping mechanism of the target gland. He suggested that the secretion of the former depends on the nervous or neurohumoral connections of the adenohypophysis, whereas that of the latter does not (Greer, 1952). This challenging concept led us to investigate critically the manner in which TSH exerts its effect on the thyroidal iodide trap and to study some of the factors which modify this action.

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[2] Present address: National Cancer Institute, Bethesda 14, Md.

MATERIAL AND METHODS

The rats used in this study were males of the Sprague-Dawley strain. Hypophysectomized rats weighing between 100 and 150 gm. were purchased from the Hormone Assay Laboratories, Chicago, maintained on a diet of Rockland pellets, horse meat and oranges, and unless otherwise stated, sacrificed 10–11 days after the operation. The non-operated animals were young adults weighing from 150 to 300 gm., which were fed Rockland pellets. TSH (USP Thyrotropin Reference Substance) was dissolved in physiological saline not more than 15 minutes prior to use. Propylthiouracil (PTU) was given either as an alkaline solution (10 mg./ml.) or as a 2% aqueous suspension with gum arabic. D,L-thyroxin was administered in slightly alkaline solutions containing 40, 200 or 400 μg. per ml. All drugs were injected subcutaneously in a volume of .5 ml. Hypophysectomized rats receiving thyroxin, as well as their controls, were given cortisone[3] (1 mg. daily) to increase their resistance to the toxic effects of thyroxin. A previous study (Halmi *et al.*, 1952) had indicated that cortisone does not have any significant effects on the iodide trap of the thyroid.

The thyroid:serum iodide concentration ratio (T/S) was determined according to the method of Vanderlaan and Greer (1950). The rats received 5 mg. of PTU solution and 45 minutes later 5 μc. of carrier-free radioiodide. One to 1½ hours after the injection of the I^{131} they were anesthetized with ether and exsanguinated via the abdominal aorta. The thyroids were dissected out and weighed on a torsion balance. Where histological studies were conducted, one thyroid lobe was fixed in Susa, embedded in paraffin and cut at 5 μ. Sections running through the center of the lobe were stained with Gomori's trichrome method and cell height measurements were made on projected slides. The other thyroid lobe was squashed on a square of filter paper placed on an aluminum planchet under a single covering layer of scotch tape. One tenth ml. of serum was pipetted onto a similar square of filter paper on a planchet, evaporated to dryness and covered with scotch tape. Thyroid and serum radioactivity determinations were carried out with a thin mica-window Geiger-Müller counter. The T/S was expressed as the calculated activity of 100 mg. thyroid tissue divided by the actual activity of the serum sample.

RESULTS

1. *Effect of hypophysectomy, thyroxin treatment and thyroxin treatment after hypophysectomy on the thyroid:serum iodide concentration ratio (T/S)*

Figure 1 summarizes the following results: a) The T/S of normal male rats was found to be very close to the value of 25:1 reported by Vanderlaan and Vanderlaan (1947); b) The T/S of hypophysectomized rats, whether determined 10 or 20 days after operation, was approximately one-fourth of the normal value; c) In rats treated with 200 μg. of thyroxin daily for 20 days the T/S was significantly lower than in hypophysectomized animals and approached 1:1; d) When 20 μg. of thyroxin plus 1 mg. of cortisone was given daily to a group of hypophysectomized rats for 5 days, starting 8 days after the operation, the T/S (24 hours after the last injection) was found to be significantly ($P < .01$) lower than in the controls which received cortisone alone.

[3] Cortisone (Cortone acetate Merck) was obtained through the courtesy of Dr. Elmer Alpert, Merck & Co., Rahway, New Jersey.

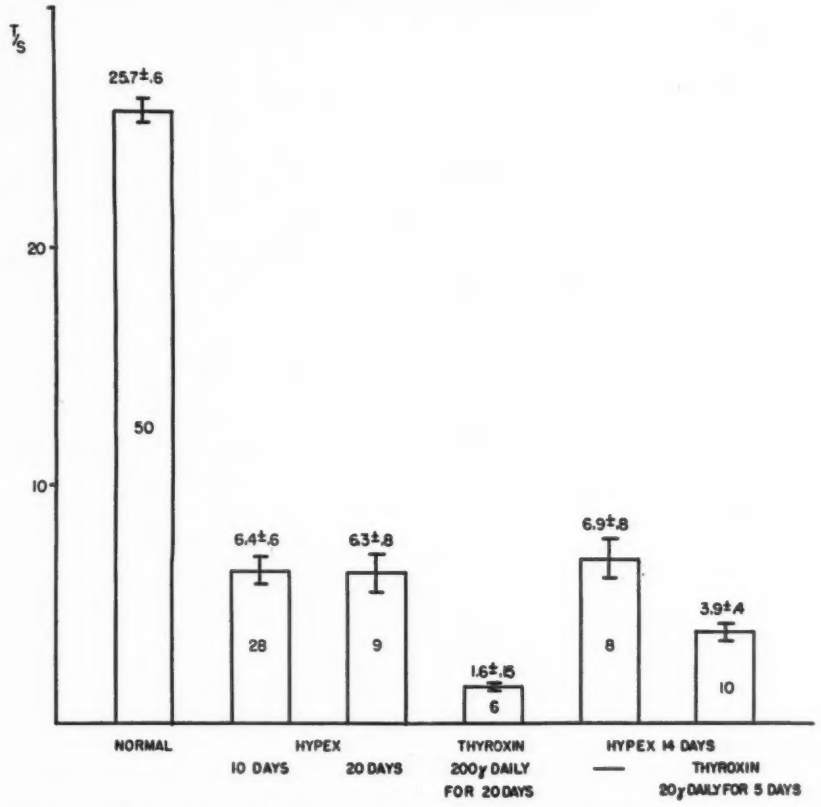

FIG. 1. Effects of hypophysectomy, thyroxin treatment, and hypophysectomy plus thyroxin treatment on thyroid:serum iodide concentration ratio (T/S). Vertical bars represent mean T/S values, vertical lines—standard errors. Figures within bars represent number of rats in group.

2. *Effect of exogenous TSH on the T/S of hypophysectomized rats*
   a) *Time curve of response after a single injection of TSH*

Figure 2 shows the change of the T/S which was observed in hypophysectomized rats following the injection of a single dose of TSH (5 mg.) 10 days after operation. The hours indicated on the abscissa were counted between the administration of TSH and the injection of radioiodide. The curve suggests a) that there was a significant elevation of the T/S 7 hours after the injections of TSH, b) that the response reached a peak between 24 and 48 hours, and c) that the effect had largely subsided by 72 hours.

Since an 8 hour latency was reported for the reaction of the iodide trap

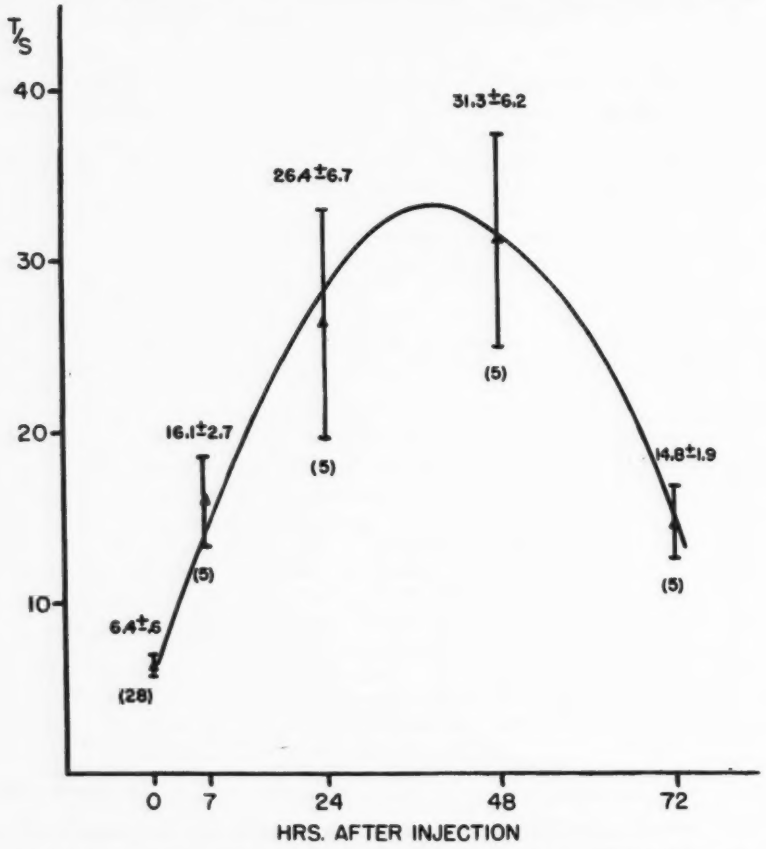

FIG. 2. Time curve (from 0–72 hours) of T/S response to 5 mg. TSH in hypophysectomized rats. Solid triangles represent mean T/S values, vertical lines—standard errors, figures in parentheses—number of rats per group.

of the human thyroid to injected TSH (Stanley and Astwood, 1949) and the effect of TSH on the discharge of radioiodine from the rat thyroid was observed to become manifest after a latent period of 2–3 hours (Wolff, 1951), the early part of the T/S time curve after the injection of a single dose of 20 mg. of TSH into hypophysectomized rats 10 days postoperatively was subjected to scrutiny. As Figure 3 shows, the first significant ($P < .01$) elevation was encountered after 8 hours, and by 10 hours after the administration of TSH the rise in the T/S was substantial. On the basis of this curve the T/S response of hypophysectomized rats to TSH appears to have a latency of around 5–6 hours.

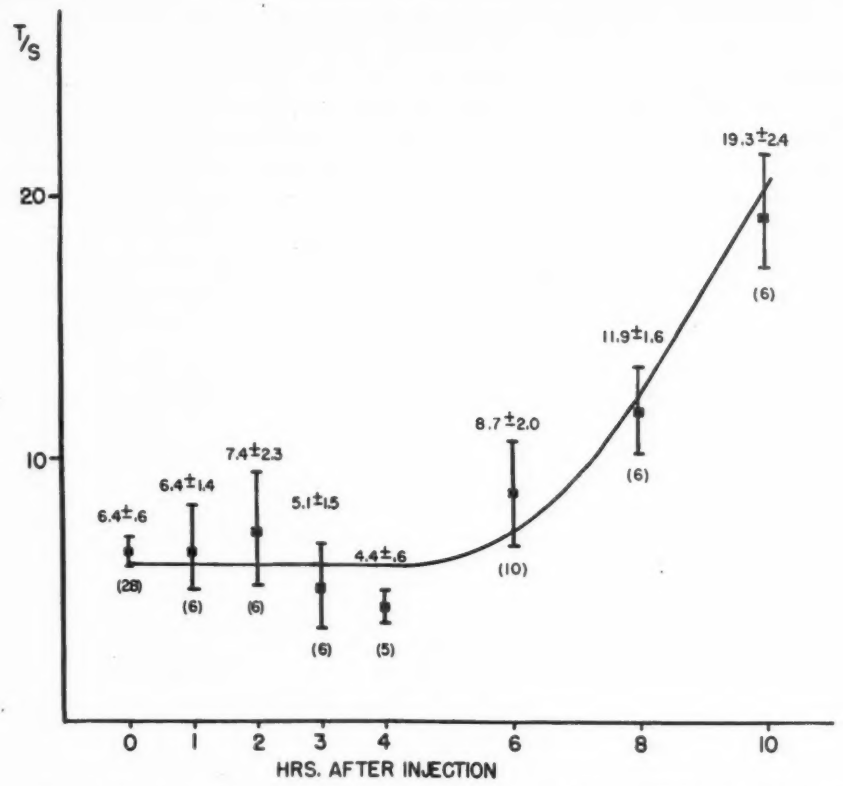

FIG. 3. Time curve (from 0–10 hours) of T/S response to 20 mg. TSH in hypophysectomized rats. Solid squares represent mean T/S values, vertical lines—standard errors, figures in parentheses—number of rats per group.

b) *Dose-response relationships. Influence of thyroxin and PTU on the effect of a single dose of TSH*

After the elevating effect of a single dose of TSH on the T/S of hypophysectomized rats had been established, a study of the effectiveness of various dose levels was undertaken. The results of these experiments are summarized in Table 1. Determinations made concurrently are listed in the same vertical column. Experiments III and X, as well as VI and VII, were carried out simultaneously. In all experiments but VIII rats hypophysectomized 10 days earlier were injected with single doses of TSH and the T/S determined 34 hours later, i.e. close to the peak of the time curve. In experiment VII 100 μg. of thyroxin was injected at the same time as the TSH, whereas in experiment IX the administration of TSH was ac-

TABLE 1. T/S RESPONSES OF HYPOPHYSECTOMIZED RATS TO GRADED DOSES OF TSH OR TSH CONTAINING TUMOR EXTRACT

Experiment No.	I ▲*	II ▼*	III ×*	IV	V	VI	VII	VIII	IX △*	X ×*
Substance given	TSH alone						TSH+ thyroxin	TSH in divided doses	TSH+PTU	Tumor extract
Dose mg.										
0	5.2± .7† (5)‡	7.6±1.5 (4)	—	6.7±1.4 (4)	—	—	—	3.9± .6 (5)	6.5± 1.5 (4)	—
.005	—	9.1±1.2 (4)	—	—	—	—	—	—	—	—
.01	—	6.7±1.5 (5)	—	—	—	—	—	—	9.3± 2.6 (3)	—
.07	—	—	—	—	—	—	—	4.0± .3 (8)	—	—
.1	—	8.7±1.1 (4)	—	—	—	—	—	—	21.1± 4.5 (4)	—
.2	13.9±2.4 (5)	—	8.4±1.2 (4)	—	—	—	—	—	—	—
.3	—	—	—	—	—	—	—	9.4± .7 (6)	—	—
.7	—	—	—	—	—	—	—	11.8±2.1 (8)	—	—
1.0	—	30.4±7.8 (5)	—	—	—	—	—	—	105.6±13.2 (9)	—
1.5	—	—	—	—	—	—	—	—	—	42.4±8.0 (4)
2.0	38.6±5.6 (6)	—	34.3±3.5 (5)	—	—	—	—	—	—	—
3.0	—	—	—	—	—	—	—	31.4±4.1 (5)	—	—
7.0	—	—	—	—	—	—	—	4.78±2.8 (8)	—	—
10.0	—	55.5±8.6 (5)	—	—	—	—	—	—	—	—
15.0	—	—	—	—	—	—	—	48.3±6.1 (6)	—	72.4±8.0 (4)
20.0	62.9±6.0 (6)	—	57.5±6.6 (5)	—	—	76.5±9.3 (8)	85.2±8.7 (9)	—	—	—
40.0	—	—	—	—	87.9±6.4 (5)	—	—	—	—	—
45.0	—	—	—	—	—	—	—	44.2±5.0 (6)	—	—
50.0	—	—	—	58.8±5.8 (7)	—	—	—	—	—	—
100.0	65.1±6.6 (5)	—	—	—	—	—	—	—	—	—
150.0	—	—	—	—	—	—	—	—	—	85.4±8.3 (5)

* Data of the various experiments included in this table are represented by these marks in Fig. 4.
† Mean± standard error.
‡ Number of rats in group.

companied by injections of 10 mg. of PTU in solution and an equal amount in suspension form. In experiment X the 34 hour response of the T/S to single injections of various doses of saline extracts prepared from TSH-containing transplanted mouse pituitary tumors[4] (Furth and Burnett, 1951, Furth *et al.*, 1952 a, b) was examined and compared with a dose-response curve simultaneously determined for the purified TSH prepara-

[4] We are greatly indebted to Dr. Jacob Furth, Oak Ridge National Laboratory, for the tumor bearing mice. The neoplasms used in this study were transplants of pituitary tumors, which had arisen after radiothyroidectomy, growing in radiothyroidectomized recipients. Extracts were prepared by homogenizing tumor tissue in saline. The hypophysectomized rats were injected with appropriate dilutions of the supernatant. The doses listed in Table 1 refer to the wet weight of tumor tissue extracted and administered per rat.

tion (experiment III). Since Ghosh *et al.* (1951) had found that a single dose of TSH was less effective in elevating the thyroid I^{131} uptake of hypophysectomized rats than the same amount of hormone given in divided doses, experiment VIII was carried out. Rats hypophysectomized 9 days previously were given 5 equal doses of TSH 12 hours apart and the T/S determined 12 hours after the lasti njection. Table 1 shows the total amount of TSH given per rat in each of the groups of this experiment.

Figure 4 is a graphic presentation of the log dose-T/S response curves obtained for TSH (showing a composite curve constructed on the basis of experiments I and II and a curve based on experiment III), for PTU and TSH (experiment IX) and for the tumor extract (experiment X).

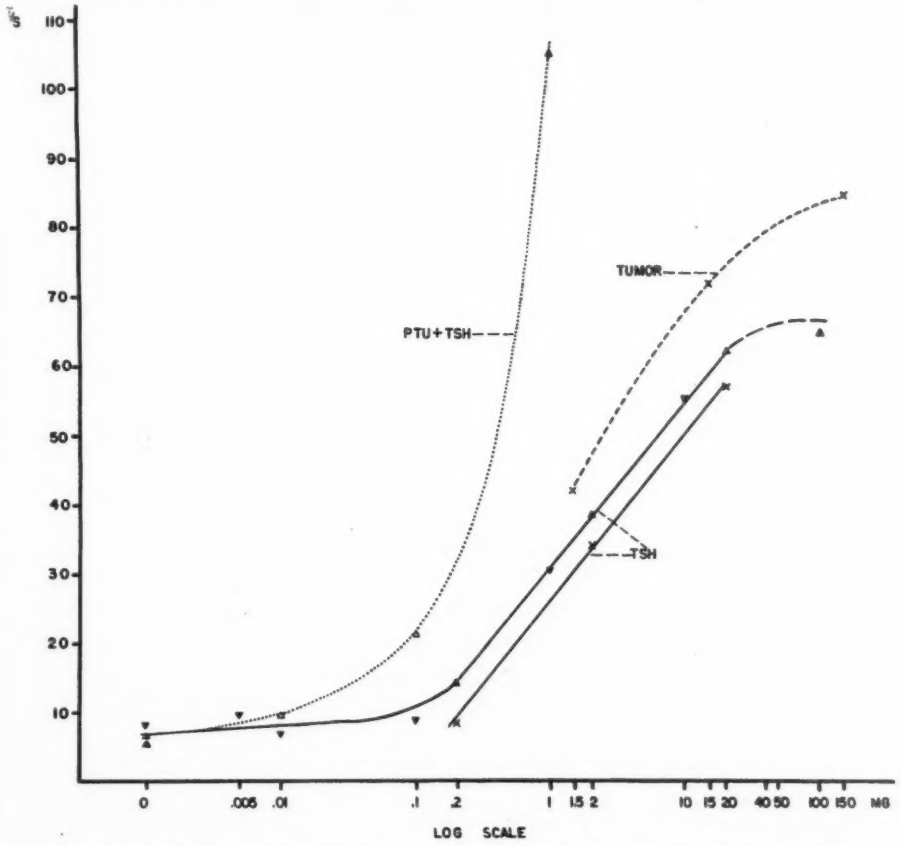

FIG. 4. Log dose—T/S response curves of hypophysectomized rats receiving TSH alone, PTU plus TSH, and TSH containing tumor extract. For further explanation consult Table 1 and text.

The quantitative data presented in Table 1 and Figure 4 can be analyzed as follows. a) The log dose TSH-T/S response curve of hypophysectomized rats appears to follow a sigmoid pattern, with the rectilinear portion lying between doses of .2 and 20 mg. Curves obtained at different times may be superimposable (experiments I and II) or at least parallel (experiment III). The great variability of responses to high doses (cf. experiments I, IV, V and VI) makes the position of the plateau shown in Figure 4 questionable. The rectilinear log dose-response relationship over a wide dosage range makes the T/S changes of hypophysectomized rats a potentially useful assay for TSH. However, the response is not sufficiently sensitive and the λ computed for the .2–20 mg. portion of the curve based on experiments I and II proved to be .489, which bespeaks relatively poor precision. b) The assay of extracts of the transplanted mouse pituitary tumors confirmed the high TSH content of these neoplasms. There appeared to be a greater concentration of extractable TSH activity in the examined sample of tumor tissue, even when expressed on a wet weight basis, than in the purified TSH preparation (cf. experiments X and III). c) One hundred μg. of thyroxin, when given together with 20 mg. of TSH, failed to have any significant effect on the T/S response to the latter (cf. experiments VI and VII. $P = .7$). d) Twenty mg. of PTU, given concurrently with single doses of TSH, significantly changed the curve of the T/S response to the hormone by increasing the sensitivity and greatly augmenting the slope for the .1 to 1 mg. dose range (experiment IX). Further studies will be necessary in order to decide whether the T/S changes in hypophysectomized rats given PTU and TSH might furnish a convenient objective method with sufficient sensitivity and precision for a practicable assay of the latter substance. e) TSH administered in divided doses was no more effective in raising the T/S than were single doses (experiments I, II, III vs. VIII). Similarly, when 10 daily injections of 1 mg. of TSH were given to 13 rats, starting 8 days after hypophysectomy, the T/S was found to be 32.7 ± 2.7[5] on the day after the last injection, which is in the same order of magnitude as the 34 hour response to a single 1 mg. dose of TSH. If there were a summation of the effects of TSH on the T/S, higher T/S values than the ones actually observed would be expected on the basis of the time curve of the response (Fig. 2) after repeated injections of the hormone.

c) *Influence of thyroxin and of PTU on the T/S response to TSH in chronic experiments*

Ingbar and Roberts (1952) stated that the effect of exogenous TSH on the T/S of hypophysectomized rats is partly counteracted if thyroxin is conjointly administered. In order to re-examine this question, we divided

[5] Mean $\pm$ standard error.

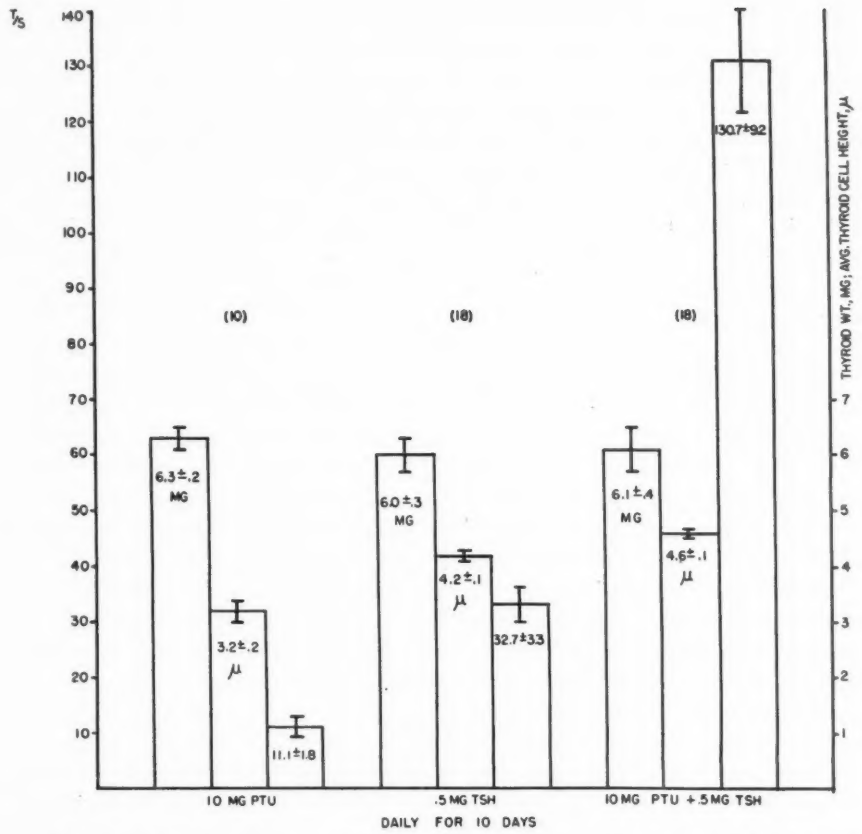

FIG. 5. Effects of PTU, TSH, and PTU plus TSH on thyroid weights, cell heights and T/S in hypophysectomized rats. Vertical bars represent mean values, vertical lines—standard errors. Figures in parentheses indicate number of rats in group, figures within bars—mean values ± standard errors.

16 hypophysectomized rats into 2 groups 8 days after the operation. Seven of the rats received 5 mg. of TSH and 1 mg. of cortisone daily for 5 days and 9 rats 20 μg. of thyroxin per day in addition. Both groups were sacrificed 24 hours after the last injection. The T/S of the former group was 36.7 ± 2.7,[5] that of the latter 39.3 ± 3.8. The difference is not significant ($P = .6$). A depressing effect of thyroxin on the T/S response to TSH could thus not be demonstrated under the conditions of this experiment.

The results of the last experiment of this study are summarized in Figure 5. Three groups of hypophysectomized rats were placed on the following regimens for 10 days, starting 8 days postoperatively: group 1 was injected

daily with 10 mg. of PTU in suspension form, group 2 received 0.5 mg. of TSH per day, and group 3 a combination of these two treatments.[6] The day after the last injection the rats were sacrificed and thyroid weights, mean acinar cell heights and T/S ratios determined. As Figure 5 demonstrates, there was no difference in thyroid weights of the 3 groups. The mean cell height was significantly higher in the TSH group than in the PTU-treated rats, and the rise in cell height brought about by the combined treatment with PTU and TSH was slightly greater than that due to TSH alone. Owing to the low standard error the difference between the cell heights of the TSH and the PTU+TSH groups was statistically significant ($P < .01$). It should be emphasized, however, that even the most stimulated thyroids in this experiment had lower than cuboidal follicular epithelium and contained large amounts of predominantly acidophilic colloid. The T/S of the rats treated with PTU only was distinctly higher than in our untreated hypophysectomized rats but less than half that of normal rats. TSH alone raised the T/S to values slightly above the normal range. TSH and PTU together had a dramatic effect, inasmuch as they caused the T/S to soar to values more than five times as high as that of intact rats. A T/S well within the "goitrous" range was thus encountered in morphologically poorly stimulated thyroids, which is a dichotomy between the histological picture and the iodide trapping function of the thyroid similar to that described by Greer (1951) in PTU-treated rats with hypothalamic lesions.

DISCUSSION

The drop of the thyroid:serum iodide concentration ratio (T/S) after hypophysectomy and its elevation to normal or higher levels by exogenous TSH is in agreement with the concept that the iodide trapping ability of the thyroid is under the influence of TSH. The finding that prolonged administration of thyroxin depresses the T/S more than hypophysectomy does confirm similar observations reported in abstract form by Ingbar and Roberts (1952). The drop in the T/S of hypophysectomized rats effected by thyroxin appears to indicate that this hormone has a direct action on the thyroid in addition to its generally accepted depressing effect on the formation of TSH by the pituitary. Cortell and Rawson (1944), studying the four hour I^{131} uptake by the thyroid in intact and hypophysectomized rats, were unable to demonstrate a depression of this function by thyroxin in the latter group. Measurement of T/S is apparently a method better suited to the study of direct effects of thyroxin on the thyroid.

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[6] The two substances were injected in different sites.

An explanation of our failure to confirm the report of Ingbar and Roberts (1952), according to which thyroxin interferes with the reaction of the T/S of hypophysectomized rats to exogenous TSH, must await a more detailed publication of their results.

The effect of PTU on the T/S is of great interest. Although Greer (1949) had found T/S values comparable to those of our untreated hypophysectomized rats in a group of rats deprived of their pituitaries and fed PTU, our results (cf. Figs. 1 and 5) seem to suggest that PTU might have a slight effect on T/S even in the absence of the anterior pituitary lobe. This question requires further investigation. The modifying effect of PTU on the curve of the T/S response to single doses of TSH is striking (Fig. 4) and may provide the basis for an objective TSH assay comparable in sensitivity and precision to those of Ghosh *et al.* (1951). Although in our experience .2 mg. of TSH was the minimum dose required to elevate the T/S of hypophysectomized rats, Ingbar and Roberts (1952) reported that .0075 mg. of TSH was the lowest effective dose. This discrepancy can probably be explained if one takes into account that these authors, as their abstract appears to indicate, treated their rats chronically with PTU prior to the injection of TSH. The experiment in which TSH was administered to hypophysectomized rats, with and without accompanying injections of PTU, is of particular interest inasmuch as it shows a spectacular potentiation by PTU of the T/S response to a dose of TSH which, both alone and together with PTU, failed to restore completely the morphological picture of the thyroid (Fig. 5). On the basis of these findings the similar dichotomy between inactive thyroid histology and "goitrous" T/S values in the cited studies of Greer (1951, 1952) lends itself to a more conservative explanation than Greer's hypothesis of two thyrotrophins. We merely have to assume that the hypothalamic lesions or the transplantation of the anterior lobe in Greer's studies deprived the adenohypophysis of its ability to react to PTU with a secretion of sufficient amounts of TSH to cause morphological changes in the thyroid. Since, however, small amounts of TSH still may have been secreted it is easy to conceive that PTU potentiated the response of the T/S to this endogenous TSH and thus led to the strikingly high T/S values.

The question arises whether, as Stanley and Astwood (1949) proposed, the effect of TSH on thyroidal iodide trapping is one of the primary functions of the hormone, which is selectively potentiated by PTU, or whether the changes which TSH brings about in thyroid iodide collection are secondary to some other alteration in thyroid iodine metabolism. Rawson (1949), observing that the increase in radioiodine collection by the chick thyroid after the administration of TSH lagged far behind the TSH-induced purge of iodine from the gland, suggested tentatively that the

primary effect of this hormone might be on iodine release and other responses of the thyroid to TSH might actually be secondary to the iodine depletion. If our problem is viewed from this angle, it should be remembered that goitrogen treatment, which raises the T/S (Vanderlaan and Vanderlaan, 1947), depletes the thyroid of iodine (Astwood and Bissell, 1944), whereas hypophysectomy, which reduces the T/S, increases the concentration of iodine in the thyroid (Chaikoff and Taurog, 1949). Furthermore, it is of interest to recall that Vanderlaan and Greer (1950) found no change in the thyroid I^{127} concentration when either PTU or TSH alone was administered to hypophysectomized rats, but a marked depletion of iodine when the two substances were given conjointly. Accordingly, in our experiments the greatest rise in the T/S occurred after combined treatment with PTU and TSH. However, although Astwood and Bissell (1944) have demonstrated that the low thyroid iodine content of thiouracil treated rats does not increase substantially after hypophysectomy, Vanderlaan and Greer (1950) have reported a rapid drop in the T/S when PTU-fed rats were hypophysectomized. It can thus be concluded that thyroidal iodide trapping can not be regulated exclusively by the iodine concentration in the gland. Another possibility is that fluctuations in the T/S are secondary and proportional to changes in the rate of iodine discharge from the thyroid, since a dependence of this function on TSH has been clearly demonstrated (Wolff, 1951, Albert and Tenney, 1951). The temporal sequence of events is not incompatible with such a hypothesis since the latency of the T/S response to TSH in hypophysectomized rats appears to be definitely longer (5–6 hours, Fig. 3) than the 2–3 hour latent period which Wolff (1951) found for the reaction of the iodine discharge mechanism. On the other hand, it has been observed that thyroid iodine loss occurs at a lower rate after hypophysectomy (Randall *et al.*, 1951) than after thyroxin treatment (Albert and Tenney, 1951), whereas in our experience the T/S is depressed more by thyroxin treatment than by hypophysectomy. However, it has to be taken into consideration that Albert and Tenney only gave 10 μg. of thyroxin daily while we obtained our marked T/S depression by 200 μg. per day. The rate of thyroid I^{131} loss measured by Wolff and by Albert and his associates was interpreted as an index of the thyroxin output. However, an accordingly rephrased hypothesis suggesting that T/S changes are secondary to alterations in the rate of thyroxin release from the thyroid is clearly untenable if one considers that the conspicuous drop of the T/S which occurs after hypophysectomy in chronically PTU-treated rats (Vanderlaan and Greer, 1950) cannot be caused by a similarly striking reduction in the rate of thyroid hormone discharge. Prolonged treatment with goitrogens largely depletes the thyroid of iodine (Astwood and Bissell, 1944) and conse-

quently leads to an eventual severe slowdown of thyroxin secretion which can hardly be further exaggerated by hypophysectomy.

In conclusion, thyroidal iodide trapping may be a function influenced by some change(s) in thyroid metabolism other than the ones discussed, or it may be directly regulated by TSH, with PTU exerting a selective potentiating effect on the response. TSH is not the only factor which affects the concentration of iodide by the thyroid, since thyroxin was found to lower the T/S even in the absence of the pituitary (Fig. 1), and the blocking influences of thiocyanate (Vanderlaan and Vanderlaan, 1947) and other monovalent anions (Wyngaarden *et al.*, 1952) on the thyroid iodide trap are well known.

SUMMARY

1. The thyroid:serum iodide concentration ratio (T/S) of normal rats was found to be approximately 25:1. Hypophysectomy lowered this ratio to roughly one-fourth (6:1), and prolonged treatment with thyroxin to values approaching 1:1. The T/S of hypophysectomized rats could be further diminished by the administration of thyroxin. Exogenous TSH elevated the T/S of hypophysectomized rats.

2. The response of the T/S of hypophysectomized rats to the injection of a single dose of TSH had a latency of around 5–6 hours, reached a peak between 24–48 hours and had largely subsided by 72 hours.

3. When the 34 hour response of the T/S of hypophysectomized rats to single doses of TSH was examined, a sigmoid log dose-response curve was obtained, with the rectilinear portion lying in the range of .2 to 20 mg. TSH.

4. Both the sensitivity and the slope of the response were enhanced when propylthiouracil (PTU) was injected together with the TSH. Thyroxin, on the other hand, had no demonstrable effect on the T/S rise elicited either by a single dose or by more prolonged administration of TSH.

5. Extracts of transplanted mouse pituitary tumors (Furth) were found to have a high TSH content as judged by their ability to raise the T/S of hypophysectomized rats.

6. No summation of the T/S response was found, when TSH was injected repeatedly.

7. Prolonged administration of TSH to hypophysectomized rats in amounts which were not sufficient to restore the normal morphological appearance of the thyroids elevated the T/S values somewhat above normal. When the same dose of TSH was given together with PTU, there was little change in the histological response of the thyroid, but the T/S-raising effect of the hormone was greatly potentiated. PTU alone had a questionable influence on the T/S of hypophysectomized rats.

8. The mechanism whereby TSH influences thyroidal iodide collection is discussed.

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THE INACTIVATION OF POSTERIOR PITUITARY ANTIDIURETIC HORMONE BY LIVER EXTRACTS[1]

JAMES H. BIRNIE[2]

From the Department of Zoology, Syracuse University, Syracuse, New York

INACTIVATION of the posterior pituitary antidiuretic hormone at some site in the body has been postulated by Ingram, Ladd and Benbow (1939) on the basis of the small amount excreted after injection of posterior pituitary preparations. The *in vitro* inactivation studies of Heller and Urban (1935) and the *in vivo* observations of Eversole, Birnie and Gaunt (1949) support the existence of such a system and indicate that the greatest activity is found in liver tissue.

The inactivation of other posterior pituitary fractions, namely, the oxytocic (Gulland and Macrae, 1933) and pressor (Møller-Christensen, 1951) by the tissues have been reported. These reports, however, have not described the nature of the inactivating system.

The present investigation was undertaken for the purpose of securing additional information regarding the nature of the system in rat liver homogenates which inactivates posterior pituitary antidiuretic hormone.

Some of these observations have been reported in an earlier abstract (Birnie, 1950) and indicate the enzymatic nature of the inactivating system.

METHODS

Homogenates of tissues were prepared immediately after their removal from animals sacrificed by a blow on the head. Prior to removal of the tissues the dorsal aorta and hepatic vein were cut and as much blood as possible was drained from the carcass. The extracts were prepared from 1.0 gram weighed samples of liver in a "Parallel Grind Homogenizer" with 10 volumes of cold distilled water. The homogenates were centrifuged and then filtered through fritted glass to remove all intact cellular material. Homogenates were adjusted to pH 6.5 by means of phosphate buffers. The destruction of posterior pituitary preparations was estimated by the effect of incubation mixtures containing known amounts of vasopressin (Pitressin) on the rate of diuresis of rats given one dose of water. (3.0 ml. per 100 sq. cm. of body surface). In the standard procedure the incubation mixture consisted of 1.0 ml. of tissue homogenate plus 100 milliunits (1.0 ml.) of vasopressin and 3.0 ml. of phosphate buffer. After incubation for 30 minutes at 37° C. 1.0 ml. of this mixture was injected intraperitoneally into test rats. The rats

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[2] Present address: Department of Biology, Morehouse College, Atlanta, Georgia.

used for this diuresis test were fasted but allowed access to water for 18 hours prior to receiving a water load by stomach tube. The rate of urine excretion was followed at 30 minute intervals for a period of 180 minutes.

Urine sodium and potassium determinations were made by means of an internal standard flame photometer.

Blood pressures were determined according to the technique described by Kersten *et al.* (1947). The instrument used was a photoelectric tensometer model FAD-1 manufactured by Metro Industries.

RESULTS

A wide variety of tissues was found to possess systems capable of inactivating vasopressin during *in vitro* incubation. In the rat the greatest activity was found in homogenates of liver tissue (Table 1).

TABLE 1. INACTIVATION OF THE ANTIDIURETIC ACTION OF VASOPRESSIN (PITRESSIN) INCUBATED WITH HOMOGENATES OF TISSUES FROM RATS

Tissue	Number animals	Percentage of administered water excreted at 90 minutes
		±S.E.
Fresh liver	39	54 ±3.53
Heated liver	26	8 ±2.83
Spleen	12	21 ±3.65
Kidney	12	22 ±4.20
Whole blood	11	12 ±3.50
Skeletal muscle	12	16 ±2.21
Untreated controls	26	52 ±1.80

The vasopressin-inactivating system appears to have the properties of an enzyme. It is thermolabile, exposure to 80° C. for 10 minutes resulting in a complete loss of activity. The active principle is nondialyzable. When liver homogenates were dialyzed against ice-cold 1.0 per cent sodium chloride solution for 2 hours there was little or no loss of vasopressin inactivating ability. Dialysis for longer periods (4 or more hours) resulted in a loss of activity (Table 2). In all reactions tested the precipitation of proteins from the extracts was associated with a loss of vasopressin inactivating

TABLE 2. INACTIVATION OF THE ANTIDIURETIC ACTION OF VASOPRESSIN BY RAT LIVER HOMOGENATES WHICH HAD BEEN SUBJECTED TO VARIOUS EXPERIMENTAL PROCEDURES

	Number animals	Percentage of administered water excreted at 90 minutes
Untreated	10	51 ±3.60
Dialyzed 2 hours	7	47 ±4.20
Dialyzed 6 hours	8	33 ±3.67
Half saturation with ammonium sulphate	8	11 ±2.10
Addition of zinc sulphate (0.0005 M)	8	10 ±4.30
Addition of copper sulphate (0.0005 M)	8	8 ±3.52

ability. This loss of activity may be due to the fact that extracts left standing for longer than four hours show some decrease in activity. When the reaction of the homogenates was adjusted to pH 5.2, activity was almost completely destroyed. Further, half saturation with ammonium sulphate results in complete loss of activity (Table 2). The addition of zinc sulphate or copper sulphate (final concentration = 0.0005 M) to the incubation mixture resulted in a complete loss of inactivating ability. During incubation maximum activity was observed between pH 6.2 and 7.0

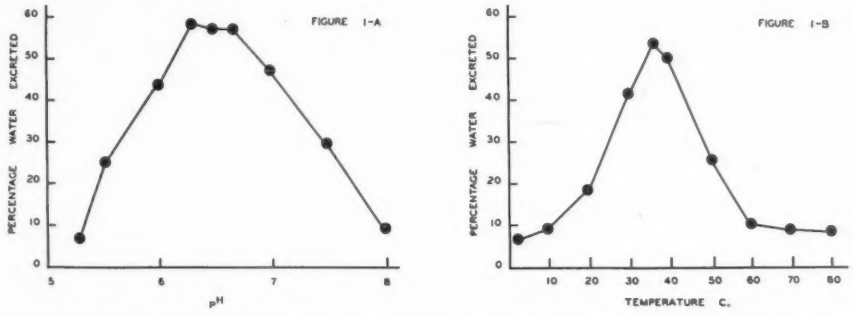

FIG. 1-A. Showing the effect on water excretion of test animals 90 minutes after injection of liver homogenates incubated at different pH levels. Each sample contained 20 milliunits of vasopressin per 0.1 gm. of tissue.

FIG. 1-B. Showing the effect on water excretion of test animals 90 minutes after injection of liver homogenates incubated for 30 minutes at different temperatures. Each sample contained 20 milliunits of vasopressin per 0.1 gm. of tissue.

(Fig. 1-A). The rate of inactivating of vasopressin increases sharply with a rise in temperature from 0° to 37° C.; however, with further increases in temperature there is a marked decline in activity (Fig. 1-B). Under optimal conditions 1.0 ml. of a 10 to 1 aqueous extract of rat liver tissue is capable of inactivating 100 milliunits of vasopressin during a 30 minute incubation period at 37° C.

No attempt has been made to determine the specificity of the vasopressin inactivating system nor to exclude the possibility that a nonspecific peptidase is involved.

It was observed that homogenates of rat liver tissues destroyed the pressor activity of vasopressin. Incubation mixtures containing homogenates of normal liver tissue failed to cause a rise in blood pressure of test rats while those made with heated liver extracts produced a marked increase in blood pressure (Table 3).

The inactivating system is not confined to tissues of the rat. Fresh human liver tissue contains a potent vasopressin inactivating system. It

TABLE 3. INACTIVATION OF THE PRESSOR ACTION OF VASOPRESSIN BY INCUBATION WITH RAT LIVER HOMOGENATES

	Number animals	Mean control blood pressure, mg. Hg	Mean blood pressure following injection, mm. Hg	Mean change in blood pressure, mm. Hg
Heated liver plus 20 mμ Pitressin	12	122	153	31
Fresh liver plus 20 mμ Pitressin	21	116	117	1
Saline plus 20 mμ Pitressin	15	122	142	20

was observed that the extracts of human liver (sample of tissue removed during abdominal surgery) not only destroyed the antidiuretic activity but also the natriuretic activity of vasopressin (Table 4).

TABLE 4. INACTIVATION OF ANTIDIURETIC AND NATRIURETIC ACTION OF PITRESSIN BY HUMAN LIVER

	Percentage water excreted at 90 minutes	Total sodium excreted at 90 minutes (meq. $\times 10^{-4}$)
Untreated controls	56.1	628
Fresh human liver (20 mμ Pitressin/0.02 g. tissue)	61.4	731
Heated human liver (20 mμ Pitressin/0.02 g. tissue)	8.4	1255

NOTE: All animals received one dose of water by stomach tube (4 ml. per 100 sq. cm. of body surface).

When extracts were made of liver taken from rats which had been given daily injections of 0.05 ml. of carbon tetrachloride in 0.05 ml. of paraffin oil for a period of 12 days, there was a sharp decline in the ability to inactivate vasopressin as compared with untreated controls (Table 5).

Extracts made of liver taken from rats which had been adrenalectomized for from 5 to 7 days showed a marked decrease in ability to inactivate the antidiuretic principle of vasopressin as compared with sham operated

TABLE 5. INACTIVATION OF THE ANTIDIURETIC ACTION OF VASOPRESSIN INCUBATED WITH RAT LIVER HOMOGENATES

Treatment of donor rats	Number animals	Percentage of administered water excreted at 90 minutes ±S.E.
Untreated	26	54 ± 2.22
CCl_4 injected 12 days	12	39 ± 3.69
Adrenalectomized 5 days	21	41 ± 2.80
Adrenalectomized 9 days	15	31 ± 2.40
Dehydrated 24 hours	10	56 ± 3.51

controls. Liver obtained from animals in the terminal stages of adrenal insufficiency showed even less activity (Table 5).

DISCUSSION

The experiments reported here show that cell-free extracts of liver tissue contain an enzyme like system capable of inactivating the antidiuretic, pressor and natriuretic principles of posterior pituitary preparations (vasopressin). The deduction of its enzymatic nature is based upon thermolability, nondialyzability, protein nature, and the kinetics of its reactions. The specificity of the system has not yet been determined. It is possible that the enzyme is a peptidase but thus far it has not been identified.

The distribution of the inactivating system in various tissues confirms the observations of Heller and Urban (1935); Jones and Sclapp (1936); and Eversole, Birnie and Gaunt (1949) on the role of the liver in destroying the antidiuretic principle of posterior pituitary extracts.

The inactivating system is not confined to the tissues of the rat as the present study demonstrates its existence in human liver and previous investigations have found it to be present in the mouse (Birnie, Blackmore and Heller, 1952) and rabbit (Møller-Christensen, 1951).

Whether the system plays a physiological role in the destruction of endogenous posterior pituitary hormone has still not been definitely established. The observation that physiological alterations cause a change in the rate of inactivation suggests that the system may play a role in controlling water metabolism in the intact animal.

It has long been postulated that water retention observed following clinical and experimental liver damage is due, at least in part, to a decreased rate of inactivation of the antidiuretic hormone by damaged liver tissue (Ralli *et al.*, 1945; Labby and Hoagland, 1947). The observations reported here are consistent with such a hypothesis. That these observations do not explain entirely the water retention associated with liver damage is suggested by the observations of Shorr and Zweifach (1948) that the damaged liver itself may produce antidiuretic materials.

It is well established that adrenalectomized animals show a deficient diuretic response to administered water; this has been ascribed in part to an accumulation of antidiuretic material in the body fluids (Birnie *et al.*, 1949; Gaunt *et al.*, 1950). It has been postulated that his increase may be due to (a) the neurohypophysis being hyperactive following adrenalectomy, or (b) a decrease in the normal destruction of antidiuretic material by the tissues. The former possibility is open to serious question on theoretical grounds (Gaunt, 1951) and the present study provides evidence of a decreased destruction of antidiuretic material following adrenalectomy. While failure of the liver to inactivate antidiuretic material

would not explain all of the alterations in water metabolism associated with adrenal insufficiency, it is possible a contributing factor to the syndrome.

SUMMARY

1. Cell free extracts of rat liver tissue contain an enzyme-like system capable of inactivating the antidiuretic, pressor, and natriuretic principles of posterior pituitary preparations (vasopressin).

2. Extracts of liver tissue inactivated vasopressin more effectively than did extracts of spleen, kidney, skeletal muscle or whole blood.

3. Extracts made of liver taken from adrenalectomized rats showed a marked decrease in ability to inactivate the antidiuretic principle of vasopressin.

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THE ACCELERATING EFFECT OF ADRENALECTOMY ON REGROWTH OF HAIR IN THE THYROIDECTOMIZED RAT

ISOLDE T. ZECKWER

Department of Pathology, School of Medicine, University of Pennsylvania
Philadelphia 4, Pennsylvania

THE accelerating effect of adrenalectomy on hair growth in the rat is a well established fact, data on which have been reviewed by Baker (1951). This is a curious phenomenon for which there is no ready explanation. If hair growth is a part of general somatic growth, it would be expected to be altered with changes in the pituitary acidophils that secrete growth hormone (somatotrophic hormone, STH). A method for depleting the pituitary of acidophil granules is by thyroidectomy, after which there is known to be retardation of the hair growth. One might expect the loss of acidophil granules in the thyroidectomized rat to prevent the accelerated growth of hair after adrenalectomy.

This hypothesis was tested by the experiments to be reported here; the results, however, were found to be the direct opposite of those anticipated. This unexpected finding was clear-cut and constant.

METHODS

For the experiments were used groups of rats of a colony of Wistar albino strain, of the same sex, usually litter-mates, sometimes non-litter mates of approximately the same age, as follows:—(1) thyroidectomized rats with their adrenals intact, (2) adrenalectomized rats with their thyroids intact, (3) rats in which both thyroidectomy and adrenalectomy had been performed, (4) normal controls.

The rats that were thyroidectomized were operated on at 29 to 109 days of age. During the course of the experiments, evidence that thyroidectomy had been adequately performed was furnished by the retarded body-weight curve and the retarded regrowth of hair after shaving. On termination of the experiment, the characteristic changes in the pituitary (basophilic thyroidectomy cells containing intracellular hyaline material, and absence or marked diminution in granulated acidophils) was the final proof of thyroidectomy.

Bilateral adrenalectomy was carried out through the usual lumbar incision 34 to 222 days after thyroidectomy, thus allowing for the slow decay period of thyroid hormone in the circulation. From the experience of previous experiments (Zeckwer 1935), a long time interval was allowed for the slow development of cellular changes in the anterior pituitary following thyroidectomy. Adrenalectomized rats were given 1% NaCl

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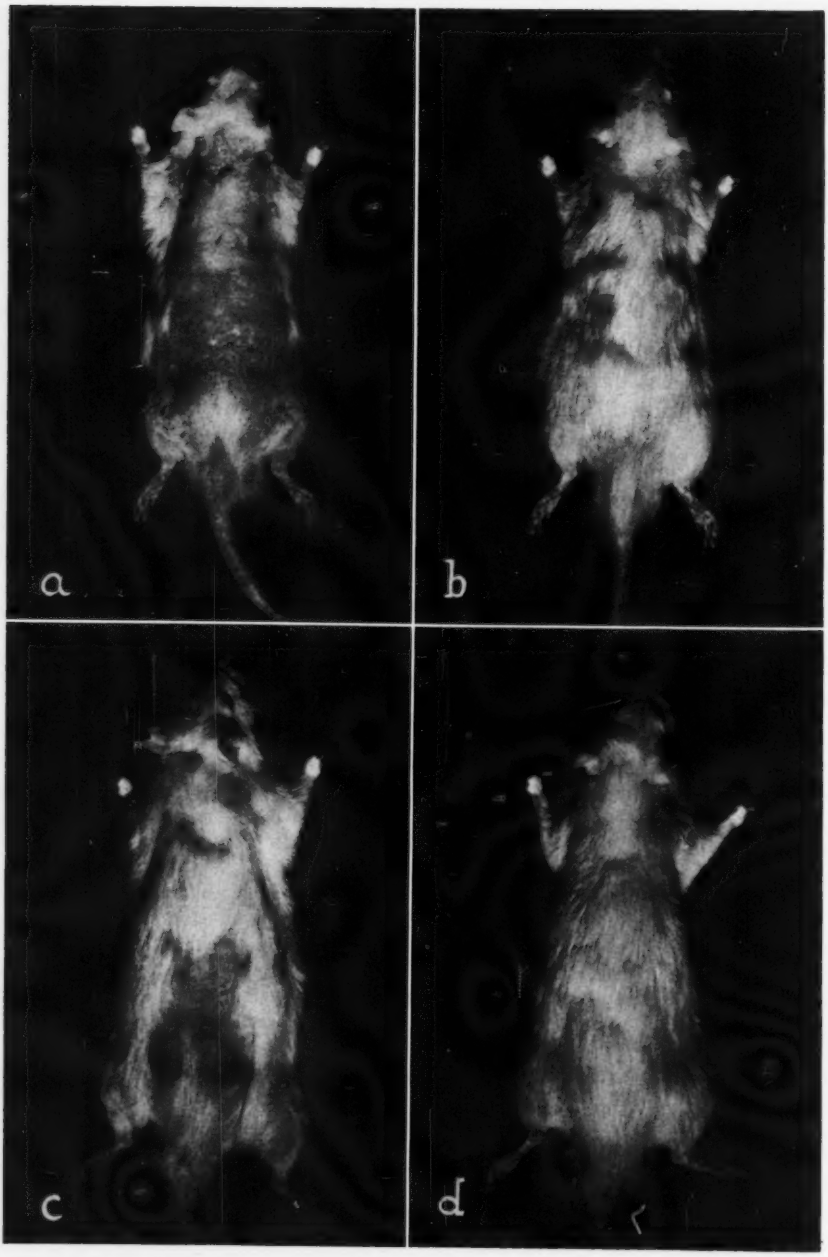
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drinking fluid. During the course of the experiments completeness of adrenalectomy in the rats with thyroids intact was attested by the much more rapid regrowth of hair after shaving, and at the termination of the experiment, proof of complete removal of adrenal tissue consisted of development of adrenal insufficiency on withdrawal of salt solution. At suitable time intervals the hair was shaved by electric clippers from the back and sides of all rats of a group, and the regrowth recorded by drawings and photographs. The same rats were shaved repeatedly, so that data could be obtained at varying time intervals before and after adrenalectomy. Periods up to 47 days after adrenalectomy were studied.

The rats were fed Purina dog chow and were kept in cages with solid sides and floors, open only at the top. Consequently the shaved rats were not exposed to draughts.

For histological studies, sections were prepared from the skin after fixation in formalin and stained with hematoxylin and eosin. The pituitaries were fixed in Helly's solution and stained with Mallory's acid fuchsin-aniline blue, orange G method.

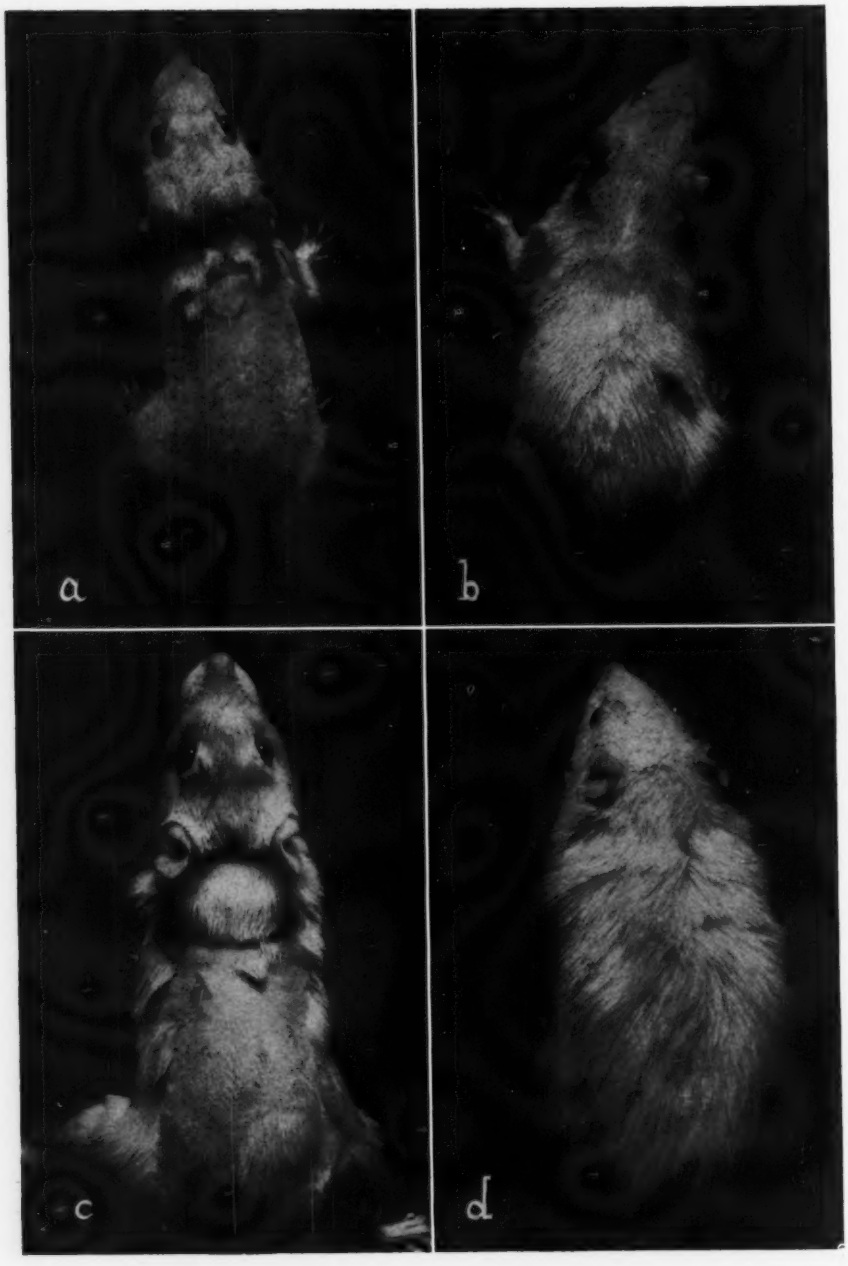
It was necessary to exclude from the analysis of the results: (a) a few very dwarfed rats thyroidectomized for a long period that survived adrenalectomy only a short time, (b) some thyroidectomized rats that had abnormalities of tooth formation which prevented adequate mastication and caused malnutrition, (c) a few adrenalectomized rats that did not develop adrenal insufficiency on withdrawal of salt. When a rat had to be excluded the remaining rats were compared with other groups of rats. In addition, sometimes rats served as their own controls when growth of hair in rats (with and without thyroid glands) was recorded before and after adrenalectomy. There remained for comparison 20 thyroidectomized rats with adrenals intact, 26 adrenalectomized rats with thyroids intact, 25 rats with combined thyroidectomy and adrenalectomy and 23 normal controls.

RESULTS

In normal control rats, regrowth of hair after shaving occurred in regular patterns and waves which have been recorded by various investigators, and details of which have been reviewed by Butcher (1934, 1951), Haddow (1945), and Wolbach (1951). This regrowth occurs symmetrically along a longitudinal axis and leaves certain areas of skin bare until the wave of growth approaches them. The location of the bare areas depends upon the time in the cycle when shaving was done. These cycles succeed each other rapidly in young rats; in older rats they progress slowly.

After adrenalectomy in rats with thyroids intact, this regular pattern of regrowth is usually not perceptible. Instead, there is a rapid diffuse

FIG. 1. Regrowth of hair, 30 days after shaving, in male litter-mate rats (49B). (a) Rat at 142 days after thyroidectomy (operation at 43 days of age). Body weight 228 grams. It can be seen that there is almost no regrowth of hair. (b) Rat at 142 days after thyroidectomy (operation at 43 days of age) and 35 days after bilateral adrenalectomy. Body weight 233 grams. In contrast to the appearance shown in the preceding figure (a) thick diffuse regrowth of hair has taken place. (c) Intact rat, showing normal patterns of hair growth, alternating with symmetrical bare areas. Body weight 288 grams. (d) Rat at 35 days after adrenalectomy. The operation was done at the same time as in rat (b). Body weight 284 grams. Thick diffuse growth of hair has occurred.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growth that occurs almost simultaneously over the entire shaved area. This effect did not appear immediately after adrenalectomy but required from 12 to 23 days for development. Once this latent period was passed, diffuse regrowth occurred rapidly after each shaving in a few days' time.

In thyroidectomized rats with adrenals intact, after about 5 weeks following operation (when thyroidectomy changes in the cells of the pituitary are extensive), the regular waves of regrowth occurred in normal patterns, but at a greatly reduced rate, and in many severely dwarfed rats that were thyroidectomized at an early age, the shaved areas remained entirely bald for long periods.

The effect of adrenalectomy in such previously thyroidectomized rats was striking and invariably the same. Adrenalectomy caused a diffuse regrowth of hair, in marked contrast to the condition of the same rat before adrenalectomy and in marked contrast to the appearance of control thyroidectomized rats with adrenals intact. However, this diffuse regrowth was much slower in development than in the adrenalectomized rats with thyroids intact. The latent period was at least one week longer. Eventually, the fur was thick and heavy and long in all adrenalectomized rats whether the thyroid was absent or intact, even in the case of extremely dwarfed long-time thyroidectomized rats that were losing weight. Figures 1 and 2 illustrate the external appearance of 2 experiments on litter-mates.

Microscopic sections of the skin of the rats show changes corresponding to the gross appearance of the hair. In the thyroidectomized rat the hair follicles are small and inactive and occur only near the surface. The dermis is very thin, and adipose tissue occurs between the muscle and dermis. In the thyroidectomized rat after adrenalectomy the follicles have become large and active and have grown deeply, down to the muscle. The dermis has thickened and adipose tissue has disappeared. The section of skin of a normal control rat, taken through a patch of hair growth, shows large active follicles. The adrenalectomized rat with thyroid intact shows good growth of many follicles.

Growth curves of the group of rats that were illustrated in Figure 2, are shown in Figure 3. It is evident that the thyroidectomized rats showed retarded growth.

FIG. 2. Regrowth of hair, 26 days after shaving, in male litter-mate rats (49R). (a) Rat 154 days after thyroidectomy (operation at 29 days of age). Body weight 172 grams. It can be seen that there is almost no regrowth of hair. (b) Rat at 154 days after thyroidectomy (operation at 29 days of age) and 26 days after bilateral adrenalectomy. Body weight 158 grams. Thick diffuse regrowth of hair has taken place. (c) Intact rat, showing symmetrical patterns of regrowth, alternating with bare areas. Body weight 282 grams. (d) Rat at 26 days after bilateral adrenalectomy. This operation was done at the same time as in rat (b). Body weight 290 grams. Diffuse thick growth of hair has occurred.

In microscopic sections the pituitary of the thyroidectomized rat and the rat with combined thyroidectomy and adrenalectomy have identical changes; namely, granulated acidophil cells have disappeared entirely in both. Each pituitary is filled with large basophilic thyroidectomy cells, many of which contain intracellular hyaline material. These thyroidectomy cells are regarded as cells producing TSH which is secreted in excess when the pituitary is released from the inhibiting effect of thyroxin.

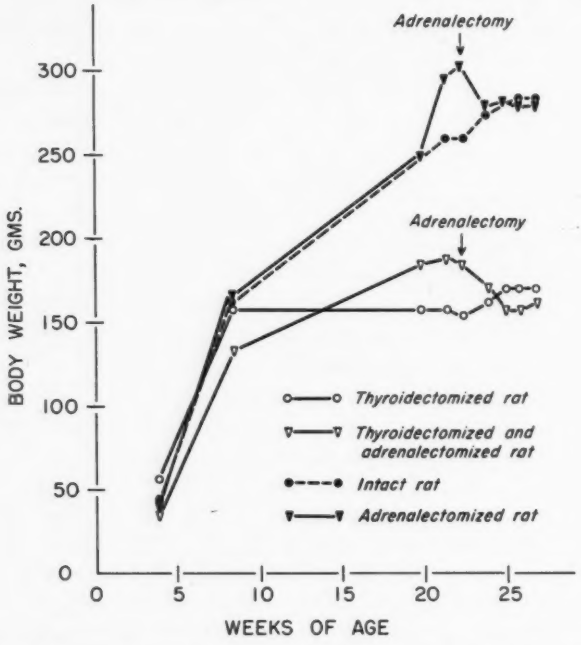

FIG. 3. Growth curve of litter-mate rats (49R) that are illustrated in Figures 2 and 3.

DISCUSSION

It is of interest to attempt a correlation of the structural with the functional changes in the pituitary. Several questions arise: Is the disappearance of granulated acidophils in the pituitary of the thyroidectomized animals a manifestation of decreased secretion of STH ? Does this mean that STH secretion is influenced by thyroid activity? Are the numerous acidophils in the pituitary of the adrenalectomized rats responsible for accelerated hair growth? Unfortunately, a simple explanation that body growth and hair growth are both manifestations of STH and are both correlated with numbers of acidophils does not hold because we find that

accelerated hair growth occurs in rats with combined thyroidectomy and adrenalectomy in which no granulated acidophils are present in the pituitary.

In spite of this apparent inconsistency, there is evidence accumulating from biochemical studies that growth hormone secretion is altered after thyroidectomy and after adrenalectomy.

That there may be an increased amount of growth hormone in adrenalectomized animals is indicated by the experiments of Engel (1951b) who states that in the adrenalectomized animal there is an increased rate of protein synthesis and a decreased rate of protein breakdown and thus presumably the adrenalectomized animal should show a greater growth rate than normal, if it were not obscured by reduced appetite of the animal. Analyses of the carcasses of normal rats restricted to the same food intake as adrenalectomized animals suggested chemical growth in the latter. He cites a thesis by J. Winternitz (1942) indicating that specific tissues may show enhanced growth rates after adrenalectomy.

Tepperman, Engel, and Long (1943) also cite Winternitz' experiments (1942) as suggesting that the removal of the adrenals which normally promote protein catabolism results in a relative hyperactivity of the anterior pituitary in promoting anabolism.

Wyman and Tum Sudan (1943) found increased growth of the epiphyseal cartilage of adrenalectomized rats, interpreted to mean that adrenalectomy releases growth hormone from the pituitary. It is of interest that they found that this increased growth of cartilage began near the end of the 2nd week after adrenalectomy, i.e. exactly at the time that elapsed before hair growth was accelerated in the present experiments.

Other biochemical studies seem to have a bearing on the question of whether growth hormone production is altered after thyroidectomy and after adrenalectomy. Hoberman (1950) in studies of amino acid and protein metabolism gives data which suggest the possibility that the secretion or synthesis of growth hormone is influenced by the activity of the thyroid gland, and he suggests that "in hypothyroidism the rapidity of breakdown of amino acids is due to a release of the catabolizing system from the inhibiting influence of growth hormone." He states that "adrenalectomy appears to be responsible for stimulation of protein synthesizing reactions whether performed on normal or hypothyroid rats" and he finds that "the nitrogen output is significantly lower in the absence of both adrenal and thyroid glands than after extirpation of either gland alone."

Is hair growth an expression of growth hormone (STH) activity? Hypophysectomy causes great retardation in regrowth of hair (Snow & Whitehead, 1935). We should not expect hair growth to be completely abolished by hypophysectomy, because, as Engel (1951a) suggests, it is generally

recognized that "hormones do not initiate or abolish any specific metabolic reaction, but simply modify the rate of existing ones." Both hair growth and body growth are suppressed by administration of ACTH or cortisone to the normal animal, so one would expect the growth of both would be increased after adrenalectomy, if the effects of growth were not masked by decreased food intake.

Van Dyke *et al.* (1950) suggest that "production of growth hormone by the pituitary is not under the control of the blood level of growth hormone." The evidence summarized in the present report suggests the possibility that the level of adrenal cortical hormone in the blood may be a means of controlling the rate of growth hormone secretion. Thus removal of the adrenals would remove an inhibition to the pituitary and release growth hormone secretion.

In the homeostatic regulation of carbohydrate metabolism, it is well established that the hypoglycemic effect of insulin secretion by the pancreas is counterbalanced by the 2 opposing factors of (1) anterior pituitary diabetogenic factor and (2) the adrenal cortical factor promoting gluconeogenesis. If the adrenal cortical factor is removed by adrenalectomy, one might anticipate that the anterior pituitary might compensate by secreting extra diabetogenic factor. Consistent with this idea is the fact that adrenalectomized rats, if given sufficient NaCl, will tend to maintain adequate levels of blood sugar. Since the anterior pituitary diabetogenic factor has been attributed to growth hormone STH, one might postulate that after adrenalectomy there might be compensatory increased secretion of growth hormone.

We should not expect accelerated hair growth to be demonstrable in such animals as dogs and cats in which fatal adrenal insufficiency occurs too rapidly.

Aside from these theoretical considerations, the observation of hair regrowth after shaving offers a very simple method for estimating the adequacy of thyroidectomy or adrenalectomy during the course of long time experiments, and may also give an estimate of rate of STH secretion even when body weight can not be taken as an index, due to inadequate food intake.

The lack of correlation between granulated acidophils and apparent STH secretion can not be explained at present. The absence of acidophil granules does not necessarily mean that no STH secretion is produced. The actively secreting cell discharges granules and then the cell reverts to the chromophobe appearance. Lack of granules may mean that there has been an accelerated rate of secretion by the acidophils to the point of exhaustion, where all hormone is discharged into the circulation and none is stored in the cell. Secretion rate and storage need not run parallel.

Whatever is the cause of the accelerated hair growth after adrenalectomy it is obvious that the changed metabolism takes several weeks to develop. This change, once established, is maintained for long periods. In the present experiment, no point was reached at which the acceleration of growth of hair disappeared.

Ralli and Graef (1945) reported that DOCA prevented the stimulating effect of adrenalectomy on regrowth of hair. At present it does not seem possible to place this finding in relation to the results of the present experiments.

A study of hair growth after combined thyroidectomy and adrenalectomy is recorded by Butcher (1941) who devised experiments to find out if accelerated hair growth after adrenalectomy was accomplished by thyroid secretion. He used very young rats, underfed so as to reduce the rate of hair growth, and thyroidectomized at 36 days of age. In some rats adrenalectomy was done at the same time, in others adrenalectomy was done 7–10 days and 3 weeks after thyroidectomy. He concluded that thyroidectomy had no effects on hair growth after adrenalectomy. However, his experiments do not extend over a sufficiently long period to give time for adequate decay of thyroid hormone in the circulation, nor could advanced pituitary changes take place. Furthermore, the periods of hair growth he studied were only 8 or 9 days. In brief, in Butcher's experiments, conditions were very different from those in the present experiments.

SUMMARY

Regrowth of hair after shaving was studied in groups of rats of the same sex and age: (1) rats thyroidectomized at 29 to 109 days of age; (2) rats thyroidectomized at the same time as in the preceding group and also adrenalectomized 34 to 163 days after thyroidectomy; (3) rats adrenalectomized at the same time as in preceding group; (4) intact controls.

Confirming previous studies, it was found that thyroidectomy retarded regrowth of hair.

In adrenalectomized rats, with their thyroids intact, there was a latent period of 12 to 23 days, after which hair grew diffusely instead of in the normal cycles, and at an accelerated rate. After adrenalectomy in previously thyroidectomized rats, there was a longer latent period of at least one week more than in those with thyroids intact, followed by a diffuse regrowth of hair that eventually equalled the growth of hair in adrenalectomized rats with thyroids intact. Microscopic appearance of skin was consistent with the gross findings.

After thyroidectomy, pituitaries showed the usual disappearance of acidophils and development of basophilic "thyroidectomy" cells. Subsequent adrenalectomy did not reverse these histological changes; that is, acido-

philic cells did not reappear. Nevertheless, from the results of other investigators, it seems likely that adrenalectomy does cause the pituitary to form more growth hormone.

In view of the fact that STH is commonly ascribed to the acidophil cells of the pituitary, the absence of acidophils in rats with combined thyroidectomy and adrenalectomy, in which accelerated regrowth of hair occurred, is an apparent inconsistency for which there is no explanation at present.

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A RAPID METHOD FOR THE QUALITATIVE AND QUANTITATIVE ESTIMATION OF THE PHYSIOLOGICAL ACTIVITY OF THE ADRENAL CORTEX[1]

ROSCOE O. BRADY, M.D.[2]

From the Department of Physiological Chemistry and Endocrine Section of the Medical Clinic, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania

IT IS apparent to investigators interested in the physiology, pharmacology, or physiological chemistry of the adrenal cortical hormones that a rapid method for the qualitative and quantitative analysis of the hormonal secretions of the adrenal cortex would be of considerable value. It is the purpose of this communication to describe such a procedure and to present preliminary data resulting from the application of these techniques.

EXPERIMENTAL

For this particular study, dogs were chosen for the experimental animal. The dogs were sacrificed by an intracardiac injection of a lethal dose of nembutal. The adrenal glands were quickly removed and placed in ice. After removing the connective tissue and fat, the tissues were blotted, weighed, and slices prepared with a Stadie slicer. An aliquot was reserved for nitrogen analysis. The remainder of the slices were incubated in a large Warburg flask containing 20 ml. of the animal's own serum along with 10 mg. of adrenal cortical stimulating hormone (Armour lot #128-105R, potency 1.6 times that of LA-1-A standard), 50,000 units of penicillin, and 0.1 gm. of streptomycin. The vessel was gassed with 95% O_2—5% CO_2; the bath temperature was 37.5° C. and the time of incubation was 20 hours.

Following incubation, the adrenal slices and incubating medium were homogenized and subjected to fractionation similar to that described by Tennent, Whitla, and Florey (1951). The aqueous homogenate was treated with 5 volumes of acetone and the precipitated proteins filtered off. After removal of the acetone by vacuum distillation, the residual aqueous phase was extracted 5 times with ethyl acetate and dried over sodium sulfate. The bath temperature for all distillations was 40° C. The ethyl acetate was

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[2] Special Research Fellow of the United States Public Health Service. Present Address: U. S. Naval Medical School, National Naval Medical Center, Bethesda, Maryland.

distilled in vacuo, the residue taken up in petroleum ether and subsequently partitioned by extracting 8 times with 30% methanol. The combined methanol portions were extracted 5 times with ethyl acetate and dried over sodium sulfate. Instead of partitioning between petroleum ether and 30% methanol, a convenient method of purifying the adrenal hormones was obtained by a slight modification of the florosil chromatography method of Nelson and Samuels (1952). The adsorbent and column were prepared according to Nelson and Samuels and the sample was placed on the column in 5 ml. of chloroform. A 25 ml. portion of chloroform was run through the column and then the steroid hormones were quantitatively eluted with 50 ml. of 25% methanol in chloroform. After removal of the respective solvents by vacuum distillation, the residue was dissolved in a minimal amount of 20% methanol in benzene and applied to a paper chromatogram prepared as described by Zaffaroni, Burton and Keutmann (1950). The descending chromatograms were run for 80 hours, the eluate evaporated under nitrogen and reapplied to a second strip which was run for 18 hours. The eluate from the second strip was evaporated and applied to a third strip which was run for 3¼ hours.

The paper chromatograms were developed simultaneously with strips run in parallel on which were placed authentic samples of cortical hormones. For the 80 hour run, 50 μg. of 17-hydroxyl-11-desoxycorticosterone (Kendall's compound E) and 50 μg. of 17-hydroxycorticosterone (Compound F) were used. Similar quantities of corticosterone (Compound B) or 17-hydroxy-11-desoxycorticosterone (Compound S) for the 18 hour and desoxycorticosterone (DOC) or 11-dehydrocorticosterone (Compound A) for the 3¼ hour run were employed as controls.

The steroids were identified by direct analysis of the paper chromatograms in an adapted Beckman spectrophotometer at 245 mμ. (Tennent *et al.* 1951). The extinction was plotted as the ordinate and distance from the origin as abscissa on graph paper and the area measured by planimetry which permitted a quantitative estimate of the amount of steroid present. Further identification was obtained by spraying the paper with 0.12% alkaline blue tetrazolium dye.[1] The steroids which have the ketol side chain reduce the dye with the production of a bluish-purple spot which can also be quantitated by measuring the absorption in the Beckman at 600 mμ. The quantitative estimation by planimetry was found to yield results in good accord with those obtained by elution and determination by the Porter and Silber method (1950) for those hormones having the 17-hydroxy ketol side chain. More specific identification of the hormones was obtained by elution from the paper chromatograms and observing the absorption

[1] E. M. Richardson, unpublished, modified from Burton, Zaffaroni and Keutmann (1950) and Chen and Tewell (1951).

spectrum from 220 to 600 mμ when dissolved in sulfuric acid as described by Zaffaroni (1950).

RESULTS

The data from eight consecutive experiments demonstrated the elaboration of a minimum of five adrenal cortical steroid hormones; viz., Compounds F, E, B, S, and A (Table 1). There was no detectable contribution of the serum to the absorption in the regions of the identified compounds. Compound F appeared to be produced in the largest quantity because after

TABLE 1. STEROID HORMONES ISOLATED AFTER INCUBATION OF ADRENAL SLICES FROM EIGHT NORMAL DOGS. MEAN VALUES WITH STANDARD ERROR OF MEAN EXPRESSED AS MICROGRAMS OF HORMONE PER MILLIGRAM OF ADRENAL NITROGEN

Compound	μg./mg. N.
F	2.6 ± .11
E	1.6 ± .06
B	.80 ± .05
A	.95 ± .02
S	.77 ± .06
Unidentified (0.035)*	1.1 ± .09
Unidentified (0.004)*	.17 ± .02

* These figures refer to two as yet unidentified compounds whose rates of descent on the paper chromatograms relative to DOC were 0.035 and 0.004 respectively.

incubating 1.46 g. of adrenal slices from a normal dog, 78 μg. of Compound F were recovered. This quantity of these particular slices contained 31 mg. of nitrogen. It is apparent that these tissues elaborated 2.5 μg. Compound F per mg. N. Direct isolation from a 1.1 g. aliquot of the same tissue without incubation yielded only 6.5 μg. Compound F. Similar large differences in values for the other steroids were found when the amount present in the original tissue was compared with that recovered following incubation.

Desoxycorticosterone was employed as the fastest moving reference compound. There was a very slowly moving component whose rate of descent on the paper chromatogram relative to DOC was 0.004. This substance reduced the blue tetrazolium dye. There was a component whose rate of descent relative to DOC was 0.035 which absorbed in the ultraviolet at 245 mμ but did not readily reduce the blue tetrazolium dye. These components have not been identified as yet. Quantitative values for these materials were estimated by comparing their extinctions with that of a known amount of Compound E.

There was some absorption of ultraviolet near the origin of the chromatogram, and there was a considerable amount of material absorbing in the ultraviolet whose rate of descent was greater than DOC. There were significant amounts of materials present in the serum which contributed to absorption at these loci.

DISCUSSION

It is clear that following the incubation of relatively small amounts of adrenal cortical tissue, significant quantities of cortical steroid hormones can be recovered by the partition and chromatographic techniques. It is quite evident that the incubating procedure is of importance in estimating the actual elaboration of steroid hormones. The largest quantity of the predominating steroids obtained by direct isolation employing similar chromatographic techniques from beef adrenal glands was 400 μg. per pound of Compounds B and A (Zaffaroni and Burton, 1951). In the present experiments, as much as 100 μg. of Compound F could be recovered after incubating 1 g. of adrenal tissue. In the studies of beef adrenal glands cited (Zaffaroni and Burton, 1951), Compound F was present in the gland in quantities considerably less than Compounds B and A. In the present experiments with dog adrenal slices, Compounds F and E were predominant. It is certainly possible that this may be due to the difference in the species of animals employed (cf. Pfiffner, 1942). The discrepancy between the quantity of hormone present in the gland and the actual physiological elaboration receives support from the experiments of Hechter *et al.* (1951) who found that the chief hormones elaborated by perfused beef adrenal glands were Compounds F and B.

It is hoped that these techniques will be useful in evaluating adrenal cortical function during various stressing conditions. Some experiments along these lines are in progress and will be reported later.

SUMMARY

A relatively rapid method for the qualitative and quantitative measurement of the steroid hormone production of adrenal cortical tissue has been described.

The following steroids have been identified and quantitative estimation of their elaboration determined: Compounds F, E, A, S, and B. Compound F is produced in the greatest quantity; Compound E somewhat less, and smaller amounts of A, S, and B were found.

It is apparent that the steroid hormones recovered after incubation differ greatly in a quantitative manner from those recovered by direct isolation from the adrenal cortex.

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A QUANTITATIVE METHOD OF DETERMINATION OF THE DIABETOGENIC ACTIVITY OF GROWTH HORMONE PREPARATIONS*

J. MAYER AND DEMETRIA N. SILIDES

Department of Nutrition, Harvard School of Public Health, Boston, Massachusetts

A NEW, rapid and quantitative method of determination of the diabetogenic activity of growth hormone preparations has been developed, based on the effect of this hormone on obese-hyperglycemic mice. The metabolic characteristics of this new Mendelian recessive condition have been described in previous publications (Bleisch, Mayer and Dickie, 1952; Guggenheim and Mayer, 1952; Mayer, Russell, Bates and Dickie, 1952; Mayer, Russell, Bates, and Dickie, 1952).

Particularly relevant to the question discussed here are the characteristics of the hyperglycemia in the obese mice. This develops as a progressive condition, usually after the 12th and before the 18th week of life. The precise time of onset seems to be conditioned by the presence or absence of genes other than "obese" in the genetic composition of the obese mice. The hyperglycemia is sensitive to the amount and the nature of the diet. It is highly sensitive to growth hormone, being either precipitated (during the prediabetic diet) or very much exaggerated (during the diabetic or hyperglycemic phase) by a single injection of growth hormone. By contrast, the blood sugar of non-obese mice are unaffected even by prolonged, massive treatment. Decreased glucose phosphorylation rates have been implicated in the etiology of the hyperphagia, in the light of the glucostatic theory of regulation of food intake (Mayer, 1952; Mayer and Bates, 1952).

Recent results (Mayer, unpublished) indicate that the obese animals are characterized by hyperfunction of the alpha cells of the pancreas and overproduction of a pancreatic hormone other than insulin. Growth hormone appears to be the trophic hormone corresponding to this new pancreatic factor.

METHOD AND RESULTS

The property of single injections or of a few injections of growth hormone to increase drastically the blood glucose of obese-hyperglycemic animals is the basis of the proposed

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method. Although reliable results were obtained with single injections in diabetic animals, maximum precision and homogeneity was obtained when using obese mice still in the prediabetic period injected for 3 consecutive days with 3 equal doses and effecting the determination on the 4th day. The Folin Ferricyanide colorimetric method (Folin,

TABLE 1. EFFECT OF THREE CONSECUTIVE DAILY INJECTIONS OF GROWTH HORMONE ON THE BLOOD SUGAR OF YOUNG HEREDITARILY OBESE-HYPERGLYCEMIC MICE

Dose (mg. growth hormone)	none (saline)	0.1	0.2	0.5	1	2	4	10
Number of animals	10	8	8	7	5	4	4	4
Mean blood sugar (mg. per cent)	160	207	218	230	248	303	355	507
Standard deviation (mg. per cent)	±20	±12	±9	±6	±3	±4	±15	±32

1930, 1932) following the Somogyi method of precipitation (Somogyi, 1945) was used. The animals were 3 to 4 months old, weighed 40 to 50 g. and were fed Purina Laboratory Chow. Sex introduces no variable in the determination.

A standard curve (given in tabular form in Table 1) was established using a usual (Armour) preparation. Doses given are the daily dosage. The results were shown to be independent of the method of preparation by comparing the standard Armour hormone with a preparation made according to the directions of Raben and Westermeyer (1951, 1952) for the isolation of "non-diabetogenic" growth hormone. (Comparison was made

TABLE 2. EFFECT OF MODE OF PREPARATION AND OF ADDITION OF OTHER PITUITARY HORMONES ON THE BLOOD GLUCOSE RESPONSE OF YOUNG HEREDITARILY OBESE-HYPERGLYCEMIC MICE

Treatment	Number of animals	Mean blood glucose and standard deviation (mg. per cent)
None (saline)	10	160 ± 20
0.2 mg. standard Armour growth hormone	8	230 ± 6
0.2 mg. Raben et al. growth hormone	6	225 ± 27
0.2 mg. growth hormone + 0.2 mg. ACTH	8	220 ± 9.2
0.2 mg. growth hormone + 0.2 mg. thyrotropic hormone	6	235 ± 15

on the basis of equal nitrogen retaining activity or growth promotion in the hypophysectomized rat.) Possible errors in the method due to contamination by other pituitary hormones were studied by determining the effect of adding daily large supplements of ACTH and thyrotrophic hormone to the standard growth hormone preparation—ACTH (Armour) even in large amounts did not modify the results. Thyrotrophic hormone (Armour) increased blood sugar values only if doses of the same order of magnitude than growth hormone were used. The preparation used (Armour) did, however, show some growth activity. (Thyroxine, in doses up to 100 μg. per animal does not increase blood sugar levels (Mayer *et al.*, in press).) These results are summarized in Table 2.

DISCUSSION

This method is simple, quantitative and speedy. It has been repeatedly used in this laboratory in studies of purification of growth hormone. It may

be emphasized again that, while the experimental conditions described represent ideal circumstances for the carrying out of the determination, it is possible, whenever available material is limited, to carry out the determination using single doses. A standard curve should be established with single doses of the reference preparation. Sensitivity, though not reproducibility, is increased when more diabetic mice (older animals) are used and when a high glucose diet is fed to the animals.

Preliminary results, indicating many similarities between goldthioglucose obesity (Waxler and Brecher, 1950) and the hereditary obese hyperglycemic syndrome suggest that goldthioglucose-treated animals, more readily available, may be used in this bioassay.

SUMMARY

A new method for the determination of the diabetogenic activity of growth hormone preparations, making use of the trophic effect of growth hormone on the blood glucose of hereditary obese-hyperglycemic mice is presented. The method is rapid and can be used with hormones prepared by different methods, even in the presence of other pituitary contaminants.

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HORMONAL FACTORS INFLUENCING FAT DEPOSITION IN THE INTERSCAPULAR BROWN ADIPOSE TISSUE OF THE WHITE RAT[1]

JEAN-PAUL LACHANCE[2] AND EDOUARD PAGÉ

From the Department of Nutrition, Institute of Physiology, Laval Medical School, Quebec, P. Q.

INTRODUCTION

IT HAS already been reported that prolonged exposure to cold leads to a hypertrophy of the interscapular brown adipose tissue in the rat (Pagé and Babineau, 1950). Since in such animals metabolism is necessarily enhanced, it was thought worthwhile to investigate the effects of hyperthyroidism on the brown fat of rats kept at ordinary room temperature. The role of adrenal hormones was also studied in view of the findings of Fawcett and Jones (1949) that the functional integrity of the adrenal cortex is essential for the normal complement of lipids in brown adipose tissue. It has also been reported that during stress, changes in the weight and appearance of this tissue parallel changes in the adrenals (Selye and Timiras, 1949; Lemonde and Timiras, 1951.)

MATERIAL AND METHODS

Young albino male rats of the Wistar strain were used. They were housed in individual cages and fed a purified ration supplemented with crystalline vitamins. This ration contained 18% protein in the form of casein and 4% fat (Mazola corn oil). Thyroid powder (VioBin), when used, was incorporated in the ration at the level of 0.05%. Hormones were administered subcutaneously in the following daily doses: thyroxine-sodium (BDH) 5 mg./kg. of body weight; cortisone acetate (Merck), 2.5 mg.; DCA (Ciba), 2.5 mg; and ACTH (Armour), 2 mg. Thyroxine-sodium was dissolved in dilute sodium hydroxide and 0.9% NaCl was used as a diluent for other hormones when indicated.

Bilateral adrenalectomy was performed in a single sitting through two cutaneous incisions under ether anesthesia. Adrenalectomized rats were given tap water containing 1% sodium chloride *ad libitum*. The animals were sacrificed by decapitation under light ether anesthesia and the interscapular brown adipose tissue was immediately dissected and weighed. It was dried to constant weight *in vacuo* at 48° C. The dry matter was later homogenized in a chloroform-methanol mixture and the fat was extracted therefrom at

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room temperature according to the method of Folch *et al.* (1951). The fresh weight of the perirenal fat (left side) was used as an index of fat depots, Pagé and Babineau (1951) having found the amount of fat in this tissue to be a reliable guide to total body fat.

Statistical treatment was limited to the determination of the standard error by the usual formula where it is equal to $\sqrt{\Sigma d^2/n\ (n-1)}$; $n(n-2)$ was used in groups numbering less than ten animals. The probability of identity between values was calculated from standard tables for Student's t.

RESULTS

Thyroid feeding

Thyroid feeding to young rats for a period of two weeks led to a considerable hypertrophy of the brown adipose tissue. This gain in the fresh

TABLE 1. EFFECT OF THYROID FEEDING ON THE BROWN ADIPOSE TISSUE OF INTACT AND OF ADRENALECTOMIZED RATS

Group	Experiment 1*					Experiment 2†	
	Intact rats			Adrenalectomized rats		Adrenalectomized rats	
	Controls (beginning of expt.)	Controls (end of expt.)	Thyroid fed	Controls (end of expt.)	Thyroid fed	Controls (end of expt.)	Thyroid fed
No. rats	10	10	10	11	10	6	7
Mean body wt., gms.							
Initial	145	145	145	145	147	236	226
Final	—	208	183	147	150	302	266
Perirenal fat, mg.	569 ± 50	942 ± 110	410 ± 60	negligible	negligible	1191 ± 300	667 ± 300
P‡		.01	.01			.7	
Brown adipose tissue							
Fresh wt., mg.	167 ± 14	284 ± 20	407 ± 21	163 ± 12	197 ± 19	350 ± 32	554 ± 69
P		.01	.01	(.9)§	.25	.02	
Water content, mg.	75 ± 5	130 ± 11	130 ± 7	108 ± 9	109 ± 6	172 ± 16	190 ± 9
P		.01	.9	(.01)	.9	.3	
Fat content, mg.	60 ± 8	113 ± 15	234 ± 20	24 ± 5	56 ± 15	124 ± 17	313 ± 26
P		.01	.01	(.01)	.05	.01	
Fat-free dry matter content, mg.	32 ± 3	41 ± 3	43 ± 5	31 ± 2	32 ± 4	54 ± 7	51 ± 13
P		.05	.8	(.8)	.8	.8	

* Experimental period: 2 weeks.
† Experimental period: 36 days; rats thyroid fed during the last 29 days.
‡ P = Probability of identity between the two values shown above.
§ Probability of identity between untreated adrenalectomized rats and controls killed initially.

weight of the tissue could be entirely accounted for by the increase in fat content since no change occurred in the water or fat-free dry matter by comparison with control rats killed on the same day (Table 1, Experiment 1). It is also seen that fat deposition in the brown adipose tissue was accompanied by a reduction of depot fat as reflected in the size of the perirenal fat. On the other hand, body growth in untreated animals results in a significant increase in all three fractions of the brown fat. This is the general picture which was invariably reproduced in all experiments of this type.

The effect of adrenalectomy and of thyroid feeding to adrenalectomized rats was studied concurrently. In the case of the non-treated animals, the

size of the brown fat at the termination of the experimental period was the same as that of control rats killed initially. Its composition was significantly altered, however, the fat content dropping markedly and being replaced by an equal quantity of water (Table 1, Experiment 1). It might be argued that the stationary weight of the brown adipose tissue was consequent upon the lack of gain in body weight of these animals. However, a similar growth inhibition of this tissue was observed in a subsequent experiment where the adrenalectomized rats registered substantial gains in body weight (Table 2). The results seem to confirm fully the conclusion reached by Fawcett and Jones (1949) and mentioned above.

TABLE 2. EFFECT OF DCA ON THE BROWN ADIPOSE TISSUE OF INTACT AND ADRENALECTOMIZED RATS

Group	Intact rats			Adrenalectomized rats	
	Controls (beginning of expt.)	Controls (end of expt.)	DCA*	Controls (end of expt.)	DCA*
No. rats	10	10	10	12	12
Mean body wt., gms.					
Initial	140	141	140	140	140
Final	—	231	226	202	218
Perirenal fat, mg.	277 ± 35	1078 ± 156	1148 ± 159	389 ± 95	549 ± 60
P		0.01	.7		.2
Brown adipose tissue:					
Fresh wt., mg.	248 ± 20	291 ± 21	291 ± 16	245 ± 18	244 ± 16
P		.2	.9		.9
Water content, mg.	—	142	126	126	99
Fat content, mg.	—	101	120	74	100
Fat-free dry matter content, mg.	—	48	45	45	45

* Administered during the last two weeks of a three week experimental period.

Thyroid feeding to adrenalectomized rats caused a slight but not significant increase in the fresh weight of the brown fat (Table 1, Experiment 1). This increase was confined to the fat fraction, however, and on this basis, it is significant at the 5% level. Identical results were later obtained in adrenalectomized rats receiving thyroxine subcutaneously (Table 3). In contrast to these findings, when larger rats were adrenalectomized and fed thyroid powder for an extended period of time, there resulted a hypertrophy of the brown fat (Table 1, Experiment 2) comparable in degree and nature to that observed in intact rats similarly treated. It is doubtful if one can exclude in such a prolonged treatment the possible intervention of accessory adrenal tissue or even the cortical-like action of androgens. These same rats were also reasonably well supplied with fat reserves in contrast to the previous ones so that in the smaller rats, limited response to thyroid

feeding or to thyroxine administration may have been partly due to lack of mobilizable fat.

Desoxycorticosterone acetate

Results shown in Table 2 indicate that the administration of DCA has no effect on the size of the brown adipose tissue either in intact or in adrenalectomized rats, nor does it affect significantly the amount of depot (perirenal) fat. Analyses of the brown fat were carried out on pooled samples so that the significance of any observed difference cannot be evaluated statistically. It may be noted, however, that in both treated groups there occurs a rise in the fat content of the brown adipose tissue and a concomitant decrease in water, a unique finding in all our studies to date.

Cortisone and thyroxine in adrenalectomized rats

The effect of cortisone in adrenalectomized rats receiving thyroxine or not was next investigated. Adrenalectomized controls received no treatment or were given thyroxine alone (Table 3). It is seen that cortisone alone caused a significant increase in the size and fat content of the brown adipose tissue as compared to the untreated controls. In fact, this hypertrophy is as large as that observed following thyroxine or cortisone treat-

TABLE 3. EFFECT OF THYROXINE, CORTISONE, AND OF THYROXINE + CORTISONE ON THE BROWN ADIPOSE TISSUE OF ADRENALECTOMIZED RATS

Group	Control	Thyroxine	Cortisone	Thyroxine + cortisone
No. rats	10	6	10	10
Mean body wt., gms.				
Initial	115	119	115	116
Final	135	125	132	115
Perirenal fat, mg.	88 ± 20	negligible	215 ± 30	112 ± 20
P vs controls			.01	.4
Brown adipose tissue				
Total fresh weight, mg.	254 ± 18	309 ± 25	403 ± 19	546 ± 42
P vs controls		.1	.01	.01
P vs cortisone group		.01		.01
Water content, mg.	152 ± 9	162 ± 6	169 ± 7	174 ± 10
P vs controls		.1	.1	.3
Fat content, mg.	62 ± 8	99 ± 17	182 ± 16	316 ± 33
P vs controls		.05	.01	.01
P vs cortisone group		.01		.01
Fat-free dry matter content, mg.	40 ± 5	48 ± 4	52 ± 6	56 ± 5
P vs controls		.1	.07	.02

Experimental period: 14 days; thyroxine or cortisone given on the 9th, 10th and 11th day.

ment in intact rats (Tables 4 and 5). When given in conjunction with thyroxine, the increase in weight and fat content is double that obtained with cortisone alone.

These results would indicate that cortisone administration restores the action of thyroxine which is otherwise greatly impeded by adrenalectomy.

Cortisone and thyroxine in intact rats

In intact animals, the action of thyroxine is similar to that obtained by thyroid feeding (Tables 4 and 5). Cortisone has an effect comparable to

TABLE 4. EFFECT OF THYROXINE AND OF CORTISONE ON THE BROWN ADIPOSE TISSUE OF INTACT RATS

Group	Control	Thyroxine	Cortisone
No. rats	20	10	8
Mean body wt., gms.			
Initial	135	137	135
Final	170	149	140
Perirenal fat, mg.	436 ± 37	288 ± 37	328 ± 50
P vs controls		.02	.1
Adrenal wt., mg.	23.7 ± 0.6	26.6 ± 0.6	19.0 ± 1.5
P vs controls		.01	.01
Brown adipose tissue:			
Total fresh wt., mg.	326 ± 19	461 ± 26	426 ± 27
P vs controls		.01	.01
P vs thyroxine group			.4
Water content, mg.	156 ± 7	157 ± 9	177 ± 7
P vs controls		.9	.05
Fat content, mg.	124 ± 12	254 ± 17	190 ± 17
P vs controls		.01	.01
P vs thyroxine group			.02
Fat-free dry matter content, mg.	46 ± 3	50 ± 4	59 ± 6
P vs controls		.4	.08

Experimental period: 6 days; thyroxine or cortisone given on the 1st, 2nd and 3rd day.

that of thyroxine with this difference that the water content rises significantly and that the deposition of fat is less pronounced (Table 4). Seifter *et al.* (1951) have also reported an increase in the size and fat content of this tissue in pregnant rats given cortisone. On the other hand, Antopol (1950) working on mice and using relatively larger doses of cortisone observed an atrophy of the brown fat. Sala *et al.* (1951) report that this tissue regresses under the influence of cortisone administered over an extended period of time.

When cortisone is given in conjunction with thyroxine, an apparently

TABLE 5. EFFECT OF THYROXINE, THYROXINE + CORTISONE AND OF ACTH ON THE BROWN ADIPOSE TISSUE OF INTACT RATS

Group	Control	Thyroxine	Thyroxine + cortisone	ACTH
No. rats	12	12	12	12
Mean body wt., gms.				
Initial	134	134	134	134
Final	174	153	132	171
Perirenal fat, mg.	411 ± 50	90 ± 10	100 ± 10	421 ± 60
P vs controls		.01	.01	.9
Adrenal wt., mg.	23.7 ± 0.7	28.1 ± 1.2	18.6 ± 1.4	25.8 ± 0.7
P vs controls		.01	.01	.08
Brown adipose tissue				
Total fresh wt., mg.	310 ± 15	441 ± 15	544 ± 29	367 ± 22
P vs controls		.01	.01	.05
P vs thyroxine group			.01	
Water content, mg.	156 ± 4	151 ± 5	161 ± 9	174 ± 9
P vs controls		0.5	0.6	0.1
P vs thyroxine group			.3	
Fat content, mg.	107 ± 11	240 ± 9	333 ± 19	134 ± 14
P vs controls		.01	.01	.2
P vs thyroxine group			.01	
Fat-free dry matter content, mg.	47 ± 1	50 ± 3	50 ± 6	59 ± 4
P vs controls		.3	.6	.01

Experimental period: 8 days; Rats injected daily, the 6th day excepted.

additive response is obtained (Table 5). It is to be noted that cortisone induces an atrophy of the adrenals even in the presence of excess thyroxine. The fact that this hormone is equally potent whether the adrenals are removed or atrophied points to its preponderant role among cortical hormones with respect to fat deposition in the brown adipose tissue.

ACTH in intact rats

The action of ACTH is reported in Table 5. The dosage used was too low to increase significantly the size of the adrenals. Nevertheless, the brown adipose tissue was significantly hypertrophied and a general increase in all three fractions occurred, the rise in fat-free dry matter content being statistically significant. This response differs somewhat from that obtained with thyroxine or cortisone and is more reminiscent of spontaneous changes occurring during body growth (Table 1). Baker, Ingle and Li (1950) have observed a higher fat content of the brown adipose tissue in rats treated for 21 days with ACTH. From the trend shown in Table 5, it can be assumed that a longer treatment would have yielded similar results.

Hormonal treatment and depot fat

With respect to general fat stores, it can be seen that hyperthyroidism led to a considerable decrease in the amount of perirenal fat in all cases. Cortisone, on the other hand, seemed to prevent the loss of fat consecutive to adrenalectomy although it did not affect body weight. It also afforded some measure of protection against fat depletion in thyroid-treated adrenalectomized rats. These results are in accordance with the findings of Stoerk and Porter (1950). In intacts rats, neither cortisone nor ACTH had a perceptible effect on depot fat and the same seems to apply to DCA in intact and in adrenalectomized animals.

DISCUSSION AND CONCLUSIONS

Of chief interest are the findings: (1) that thyroxine elicits a minimum response in the absence of the adrenals; (2) that cortisone is equally effective in intact and in adrenalectomized rats; and (3) that an enhanced response is obtained when the two hormones are combined either in intact or adrenalectomized animals. There thus appears to exist a synergism between cortisone and thyroxine in respect to fat deposition in the brown adipose tissue. It may be that excess thyroxine given alone exhausts the cortisone producing capacity of the adrenal while cortisone given singly inhibits the production of thyroid hormone as some reports indicate (Money *et al.*, 1951; Lederer, 1952).

The origin of this extra fat in the brown adipose tissue remains to be determined. Experiments now underway seem to indicate that it is mobilized from fat stores in other regions. In view of the reported antilipogenic action of cortisone, one would hardly expect this fat to have been synthesized *in situ*. It is difficult to correlate our findings with those of Engel and Scott (1951) regarding the effect of cortisone on glycogen deposition in the brown fat. These authors express the glycogen concentration on a fat basis and should cortisone have stimulated fat deposition, it would follow that glycogen concentration would suffer an apparent decrease unless the whole tissue content in this substance rose proportionately.

Glycogen deposition in the brown adipose tissue is often considered as indicative of fat synthesis (Wertheimer and Shapiro, 1948). However, if the fat laid down in the brown adipose tissue originates elsewhere, one may ponder whether it is later oxidized directly or over the pathway of carbohydrate synthesis. Ingle (1950) has envisaged the possibility of fat conversion to carbohydrate under the influence of corticosteroids and Segaloff and Many (1951) suggest that extra urinary glucose in phlorizinized rats may have arisen from fat. ACTH and cortisone were among the compounds having a marked effect on gluconeogenesis and cortisone was much less

ketogenic than other such substances. Wells and Kendall (1940), in similar studies, had already reported a synergism between the actions of Compound E and of the thyroid gland so that the full exhibition of either was dependent upon the presence of adequate amounts of the other hormone. The similar interrelationship between cortisone and thyroxine in respect of gluconeogenesis and of fat deposition in the brown adipose tissue is striking although no link between the two processes has been shown to exist.

SUMMARY

1. Thyroxine and cortisone both promote the accumulation of fat in the brown adipose tissue of intact rats and their actions appear to be synergistic. Little or no change occurred in the water and fat-free dry matter contents.

2. In adrenalectomized animals, the response to thyroid hormone was minimal while the action of cortisone administered alone or in conjunction with thyroxine was comparable to that obtained in intact rats.

3. The administration of DCA caused no change in the size of the brown fat.

4. ACTH caused a slight hypertrophy of the brown adipose tissue, characterized by increases in all three fractions studied.

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A SODIUM-EXCRETING EFFECT OF DESOXYCORTICOSTERONE IN ADRENALECTOMIZED MICE*

BRUCE T. FORSYTH, CAPT. M.C., U.S.A.†

Army Medical Service Graduate School, Walter Reed Army Medical Center, Washington 12, D. C.

METHODS recently reported for the bioassay of desoxycortisosterone (DOC) (Dorfman, 1947; Dorfman, 1949; Deming and Luetscher, 1950; Spencer, 1950; Simpson and Tait, 1950, 1952: Luetscher and Demng, 1951; Marcus, Romanoff and Pincus, 1952) have utilized the action of DOC in adrenalectomized animals in decreasing urinary excretion of sodium, in increasing the urinary excretion of potassium or in effecting a combination of these actions. Little quantitative information has been published regarding the optimal sodium loading of the animals used for bioassay. Workers employing isotopic techniques have favored a low sodium load (Dorfman, 1947; Simpson and Tait, 1952) while those using the alteration in the urinary excretion of sodium as determined chemically or by flame photometer have favored a high salt load (Luetscher and Deming, 1951; Spencer, 1950; Marcus, Romanoff and Pincus, 1952). Although much is known concerning the effect of water loading in adrenalectomized animals (Gaunt, 1944, 1950; Hays and Mathieson, 1945; Birnie, Eversole and Gaunt, 1948; Frost and Talmage, 1951) few of these results can be applied to determining the optimal water load for the bioassay of salt retaining steroids, because urinary sodium excretion was not adequately measured.

The present studies were initiated to define more precisely the optimal sodium and water loads in adrenalectomized mice used for the bioassay of DOC. During the course of the sodium loading experiments, DOC was found to have a sodium excreting effect in small doses, and the usual sodium retaining effect in larger doses. Strain differences in sensitivity to DOC were noted in respect to both sodium excretion and sodium retention.

METHODS

Method 1

Male mice, strain CF1 Carworth Farms and strain C57 BBF1 Roscoe B. Jackson

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* From the Metabolism Division, The Department of Medicine; Washington University School of Medicine, St. Louis, Missouri

† Fellow in Metabolic Diseases. Present Address: Walter Reed Army Medical Center, Washington, D. C.

Memorial Laboratory, weighing from 20 to 25 grams were adrenalectomized using a single dorsal skin incision. These mice were maintained postoperatively on 0.9% sodium chloride solution, hereafter called saline, and "Friskies" dog biscuit containing 2.8 mg. sodium and 4.8 mg. potassium per gram of biscuit. Tests were performed from the fourth to the fourteenth post-operative day. Sixteen hours before assay solid food was removed and the animals were given a 10% solution of glucose or sucrose to drink. Desoxycorticosterone (free alcohol) dissolved in 10% ethanol was used throughout these studies. The control mice received 0.2 ml. 10% ethanol intraperitoneally, approximately 20 minutes before the assay was begun. The appropriate dose of sodium loading solution was calculated on the basis of body weight and injected subcutaneously using a number 25, two inch needle. In addition to sodium chloride the loading solution contained 5% glucose and four m.eq. per liter potassium. Urine was forcibly expressed by manual pressure over the bladder and the animals were placed in individual 600 ml. beakers with $\frac{1}{4}$ inch wire screen platforms over the bottom of the beakers. At the end of 5 hours the animals were removed and any feces present were discarded. Urine was again forcibly expressed from the bladder into the beakers. The contents of the beakers were transferred to volumetric flasks using four washes of distilled water. The total amounts of sodium and potassium in the urine were then determined with the Weichselbaum-Varney flame photometer.

Method 2

It was noted that the physical condition of the mice was often poor using Method 1 and the mortality during assay occasionally was 10–20%. For this reason the following modification was investigated. Nineteen hours before assay solid food was omitted and a solution of 10% glucose in 0.9% saline was used for drinking. Three hours before assay the mice were given only 10% glucose to drink. During the 5 hour assay period a cube of table sugar was placed in each beaker. Strain BBF1 mice ate the sugar very well, but strain CF1 mice largely ignored it. The sugar used contained approximately 0.06 mg. of sodium and 0.01 mg. of potassium per gram. In view of the small amount of sugar contaminating the urine, this source of error can be overlooked.

RESULTS

Strain CF1 Mice, Method 1

Mice of this strain were given loading solutions containing 0.5 mg., 0.3 mg., 0.1 mg. and 0.025 mg. of sodium chloride per gram of body weight administered subcutaneously in a volume of 0.5 ml. To observe the effect of the volume of loading solution on sodium excretion and DOC sensitivity the 0.5 mg. per gram, 0.1 mg. per gm. and 0.025 mg. per gram doses of sodium chloride were also injected in a volume of 2 ml. The excretion of sodium per gram of body weight under these experimental conditions with and without DOC are presented graphically in Figure 1. The decrease of urinary sodium produced by DOC was most evident with 0.5 mg. per gram of weight in a volume of one half ml. With this load a significant decrease in sodium excretion was apparent with 10 μg. of DOC ($P = 0.007$). When the same amount of sodium chloride was given in a volume of 2 ml. a dose of 25 μg. of DOC was required to establish a significant decrease in sodium

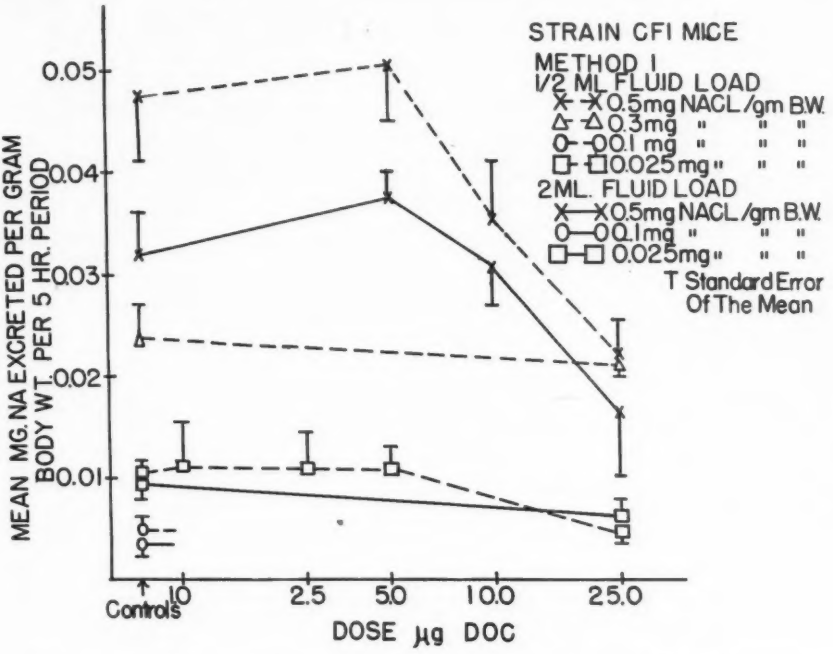

FIG. 1. The urinary sodium excretion in the strain CF1 mouse and its response to desoxycorticosterone with various fluid and NaCl loads. Standard error of the mean indicated in only one direction to avoid overlap. Each point represents the mean of from 8 to 35 animals.

excretion. With sodium loads less than 0.5 mg. of NaCl per gram of weight the control sodium excretion was correspondingly reduced. The absolute decrease in sodium excretion following DOC under these circumstances was small and too variable to be useful for assay. From these results pretreatment with 0.5 mg. NaCl per gram of body weight in 0.5 ml. was the most favorable condition for demonstrating the salt-retaining action of DOC.

Strain CF1 Mice, Method 2

Allowing adrenalectomized mice access to saline drinking water until 3 hours before assay in effect increases the salt load. The excretion of sodium with and without DOC under these conditions is presented graphically in Figure 2. Urinary sodium actually increases with doses of 5 and 10 µg. of DOC. Not until a dose of 25 µg. of DOC was given did a fall in urinary sodium from the maximum value occur. A statistical analysis of these results is presented in Table 1.

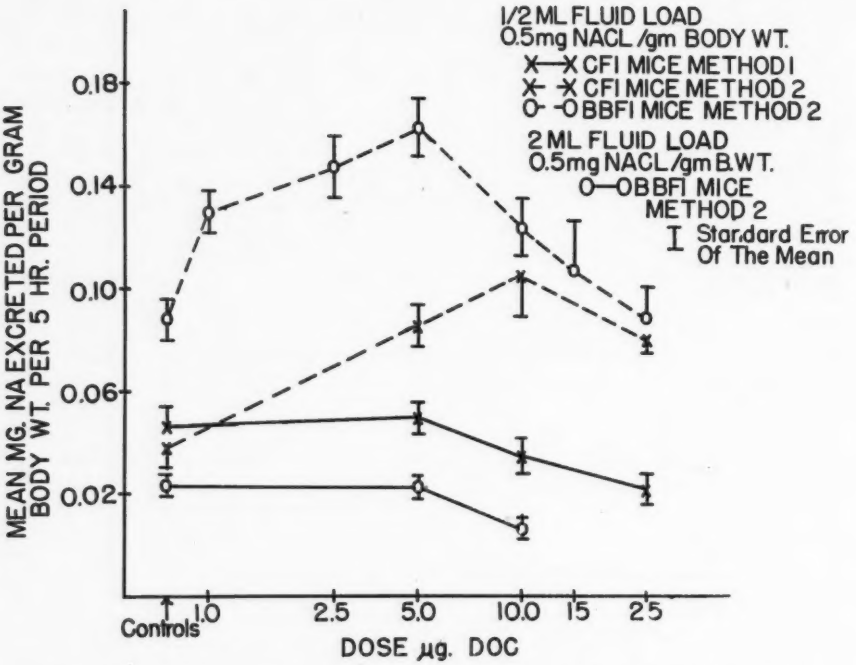

FIG. 2. The comparative effect of desoxycorticosterone on the urinary sodium excretion of strain CF1 mice and strain BBF1 mice. Standard error of the mean indicated in only one direction at times to avoid overlap. Note different scale from figure 1.

Strain BBF1 Mice

To find mice more sensitive to DOC than the CF1 strain, mice of the C57 BBF1 strain were tested. These mice tolerated sodium restriction very badly and when tested with Method 1, the mice were in such poor condition that sodium excretion was always very low regardless of fluid or sodium load used. For this reason studies on this strain are limitd to Method 2. With a sodium load of 0.5 mg. NaCl per gm. body weight and a fluid load

TABLE 1. THE SODIUM EXCRETING AND RETAINING EFFECT OF DESOXYCORTICOSTERONE IN STRAIN CF1 MICE

	Controls	5 µg. DOC	10 µg. DOC	25 µg. DOC
No. of Animals	8	13	11	8
Mean mg. Na Excr. per gm. body wt. per 5 hrs.	0.0386	0.0857	0.1053	0.0806
Stan. error of mean	±0.0087	±0.0086	±0.0150	±0.0059

of 0.5 ml. DOC had a pronounced sodium excreting effect (Fig. 2). Maximal sodium excretion occurred with 5 μg. DOC in contrast to the strain CF1 mice which required 10 μg. DOC for maximal sodium excretion. A nearly linear fall in sodium excretion occurred when plotted against the

TABLE 2. THE SODIUM EXCRETING AND RETAINING EFFECT OF DESOXYCORTICOSTERONE IN STRAIN BBF1 MICE

	Control	1 μg. DOC	2.5 μg.	5.0 μg.	15 μg.	15 μg.	25 μg.
No. of Animals	22	10	8	20	20	8	11
Mean mg. Na excr. per gm. body wt. per 5 hr. period	0.0884	0.1309	0.1480	0.1635	0.1256	0.1083	0.0897
Standard error of mean	± .0088	± .0083	± .0137	± .0102	± .0112	± .0192	± .0138

logarithm of the dose at levels of 10 μg., 15 μg. and 25 μg. doses of DOC in the BBF1 mice. (The numerical values are analyzed in Table 2.) When the same amount of sodium chloride was given in a volume of 2 ml. (Fig. 2) no increased excretion of sodium occurred with small doses of DOC. The promotion of sodium excretion by 5 μg. of DOC decreased markedly with increasing fluid load (Fig. 3).

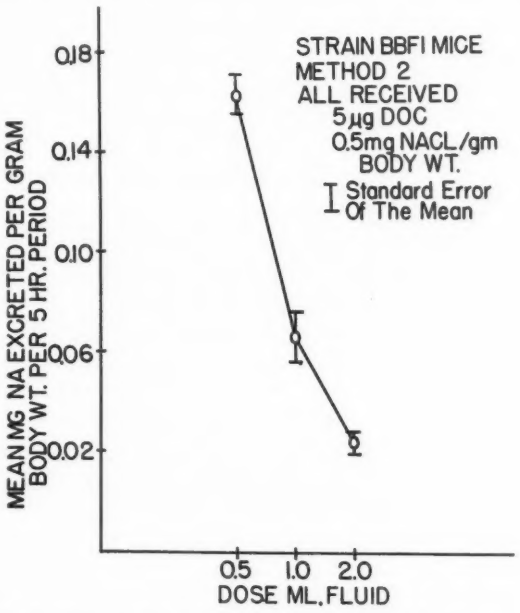

FIG. 3. The effect of increasing fluid load on the maximal urinary sodium excretion of BBF1 mice produced by 5 μg. DOC. Each point represents the mean of from 7 to 20 animals.

To minimize intestinal absorption of exogenous sodium, the drinking water containing sodium chloride was removed 5 hours rather than 3 hours before study. The control sodium excretion was somewhat lower in these experiments with a mean of 0.0601 mg. per gm. body weight per 5 hours, but increased excretion of sodium again occurred with 5 μg. DOC, the mean being 0.1006 mg. per gm. body weight per 5 hours.

Not only did the total amount of sodium but also the concentration of urinary sodium increase in strain BBF1 mice given 5 μg. DOC. Thus at the beginning of the assay 3 hours after removal of saline drinking water, the mean sodium concentration of the urine was 243 m.eq. per kilogram of urine (S.E. = ±41). Three hours after receiving 5 μg. DOC the mean sodium concentration of the urine was 424 m.eq. per kilogram of urine (S.E. = ±45). This difference is significant (P = 0.01). Urinary excretion of potassium was not significantly altered by doses of DOC up to 25 μg.

DISCUSSION

Speirs *et al.* (1951) were able to demonstrate that the strain of mice C57 BBF1 was about ten times more sensitive to cortisone as judged by eosinophil response than other strains tested. Our results indicate a comparable sensitivity to DOC in this same strain. These mice require 5 μg. DOC to reach maximal sodium excretion while strain CF1 mice required 10 μg. DOC for maximal sodium excretion. Significant sodium retention occurred in strain BBF1 mice with 10 μg. DOC, but strain CF1 mice required 25 μg. DOC for significant sodium retention. It would appear that mice of the C57 BBF1 strain are two to three times more sensitive to DOC than the mice of the CF1 strain.

The use of the C57 BBF1 strain for the bioassay of DOC seems possible under the conditions specified because of the wide differences in urinary sodium with doses of DOC from 1 μg. to 25 μg. The biphasic character of sodium excretion with DOC would necessitate testing several dosage levels of the material to be assayed in order to locate the value on the ascending or descending slope of the curve of urinary sodium excretion.

The promotion of sodium excretion in our experiments was unexpected. However, desoxycorticosterone has been shown to cause sodium excretion in Cushing's syndrome (Soffer, Gabrilove and Jacobs, 1949) and in normal dogs receiving high salt loads (Green, Farah and Klemperer, 1950). Large doses of desoxycorticosterone glycoside intravenously in humans with presumably intact adrenals causes sodium excretion, but with a fall in urine sodium concentration (Green *et al.*, 1950). The nearly twofold increase in control sodium excretion effected by 5 μg. DOC in adrenalectomized mice of the BBF1 strain could be largely attributed to increased urinary sodium concentration. Gastrointestinal absorption of sodium ap-

pears to contribute little to the sodium diuresis produced by DOC. Sodium excretion with 5 μg. DOC increased approximately to the same degree after animals had been off saline drinking water for 5 hrs. Increasing the subcutaneous fluid load decreased or abolished the sodium diuresis produced by DOC.

It is known that dogs (Roemmelt, Sartorius and Pitts, 1949) and rats (Friedman, 1948; Lotspeich, 1949) maintain a normal glomerular filtration rate for a period of time after adrenalectomy when adequately hydrated. Animals used in this study had free access to saline solution and were used for a maximum period of seventeen days after adrenalectomy. It is suggested, therefore, that increasing glomerular filtration rate was probably not the mechanism whereby DOC caused increased sodium excretion in the present experiment. Adrenalectomized dogs and patients with Addison's disease have a reduced capacity to excrete sodium under a high sodium load due to increased tubular reabsorption of sodium (Roemmelt, Sartorius and Pitts, 1949; Burnett, 1951). The sodium diuresis in the present experiments would seem to be due to a depression of tubular reabsorption of sodium with small doses of DOC, thus restoring to the adrenalectomized animal a more normal ability to excrete high sodium loads. As the dose of DOC is increased the depression of tubular reabsorption of sodium with small doses of DOC is followed by the more familiar increase in tubular reabsorption of sodium. The data at hand do not permit elucidation of the mechanism of depression of tubular reabsorption of sodium by DOC.

SUMMARY

The optimal conditions for demonstrating the action of DOC in adrenalectomized mice have been studied. A sodium load of 0.5 mg. NaCl per gm. of body weight proved most satisfactory. DOC in small doses promoted sodium excretion under high sodium loads while sodium retention occurred with larger doses of DOC. Adrenalectomized mice of the BBF1 strain are two to three times more sensitive to DOC than mice of CF1 strain.

The increased sodium excretion with small doses of DOC could be accounted for by increased urine concentration of sodium. The sodium diuresis can best be explained by decreased tubular reabsorption.

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EFFECTS OF ANDROGENS ON DEHYDROGENASE SYSTEMS

SUMNER M. KALMAN

From the Department of Pharmacology and Therapeutics, Stanford University School of Medicine, San Francisco, California

WE RECENTLY reported an inhibitory effect on the succinoxidase system of rat liver homogenates by certain water-soluble androgens (Kalman, 1952). There was some evidence in this work that succinic dehydrogenase was the target of the inhibitor. Because different dehydrogenases respond similarly to a number of inhibitors it was decided to try the effect of the androgens on the malic dehydrogenase system. In addition certain observations regarding kinetics of succinoxidase inhibition are included in this report.

METHODS

Adult white rats of the Slonaker-Wistar strain were used. No sex differences in regard to the work reported were noted and males and females were used interchangeably. The animals were killed by decapitation, and appropriate amounts of liver tissue were removed, weighed, and homogenized in cold, distilled water. Homogenates were prepared as described by Potter and Elvehjem (1936). For each enzyme system control runs were made to ensure that optimal amounts of all components were present. Control QO_2 values fell within the range of conventional results. Calculations of oxygen consumption were based on time periods during which control vessels showed essentially linear oxygen uptake. The gas phase for all systems was oxygen. In addition to the 3.0 ml. of reaction mixture, 0.1 ml. of 5% KOH was placed on filter paper in the center well of each vessel.

For the malic dehydrogenase assay the method of Potter (1946) was used. The Warburg vessels contained 0.3 ml. each of 0.5 M malate, 0.5 M glutamate, 1% diphosphopyridine nucleotide (DPN) solution, 0.0004 M cytochrome C, 0.8 ml. of 0.1 M PO_4 buffer at pH 7.4, inhibitor and homogenate in amounts specified below, and distilled water to make 3.0 ml. The DPN was added from the sidearm at the start of the reaction. Nicotinamide was omitted because it did not enhance oxygen consumption over the time periods employed.

The succinoxidase system used was that described in Umbreit's text (1949). The reaction vessel contained 0.3 ml. each of 0.004 M $CaCl_2$, 0.004 M $AlCl_3$, 0.0001 M cytochrome C, 0.5 M succinate, 1.0 ml. of 0.1 M PO_4 buffer at pH 7.4, and inhibitor as described below. A 5% liver homogenate was prepared in the usual way and then centrifuged for 10 minutes at 600×gravity. Homogenate from the supernatant was added to reaction vessels which had been filled with the other reactants and chilled for several minutes.

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The androgens used were sodium androsterone sulfate, sodium testosterone sulfate, and testosterone 17-β diethylaminoethylcarbonate hydrochloride ("soluble" testosterone). These steroids were used in aqueous solutions. The sodium testosterone sulfate and sodium androsterone sulfate are water soluble at pH 7.4. Testosterone 17-β diethylaminoethylcarbonate hydrochloride is insoluble at pH 7.4 (the pH of the reaction vessels) and above, forming a very fine suspension in an aqueous medium. This suspension may be dissolved by bringing the pH back to neutrality or to the acid side. During the course of this work some diethylaminoethanol hydrochloride was made available through the courtesy of Dr. E. Oppenheimer. This compound was tested for possible *in vitro* effect since it forms the major part of the side chain in "soluble" testosterone.

RESULTS

Inhibition of the malic dehydrogenase system by small concentrations of "soluble" testosterone is shown in Table 1. Sodium testosterone sulfate

TABLE 1. EFFECT OF WATER SOLUBLE ANDROGENS ON THE MALIC DEHYDROGENASE SYSTEM OF RAT LIVER

(QO_2 = c.mm. of oxygen uptake per mg. dry tissue per hour at 37.5° C.)

Homogenate mg. dry weight per vessel	Molar concentration		QO_2	Per cent inhibition
	Sodium testosterone sulfate	"Soluble" testosterone		
1.5	—		98	Control
1.5	3.4×10^{-4}		82	16.3
1.5	6.8×10^{-4}		46.8	52.3
1.5	1.0×10^{-3}		26.0	75.0
1.5	1.4×10^{-3}		14.0	86.0
1.5	1.7×10^{-3}		16.0	84.0
3.0		—	105	Control
3.0		1.8×10^{-5}	100	4.8
3.0		2.7×10^{-5}	90	14.2
3.0		3.6×10^{-5}	46	56.0
3.0		4.5×10^{-5}	23	78.0
3.0		5.4×10^{-5}	21	80.0
3.0		6.3×10^{-5}	17	84.0
3.0		7.2×10^{-5}	12	88.5

proved to be much less effective an inhibitor as indicated by the concentrations required. Sodium androsterone sulfate was not available for test on the malic dehydrogenase system.

In Table 2 data are presented which show inhibition of the succinoxidase system by "soluble" testosterone, by sodium androsterone sulfate, and by sodium testosterone sulfate. The inhibitory effect of these compounds as shown by the concentrations used was most pronounced with "soluble" testosterone, less with sodium androsterone sulfate, and least with sodium testosterone sulfate. Diethylaminoethanol hydrochloride does not inhibit succinoxidase in concentrations of 3.5×10^{-5} molar. Such amounts are equivalent to the amount of this compound which is present in the side chain of "soluble" testosterone at concentrations of 9×10^{-5} molar.

TABLE 2. EFFECT OF WATER SOLUBLE ANDROGENS ON THE SUCCINOXIDASE SYSTEM OF RAT LIVER

QO_2 = c.mm. of oxygen uptake per mg. dry tissue per hour at 37.5° C.
All vessels contained 3.0 mg. dry weight of homogenate.
Concentrations of drugs are expressed in final molar concentration

Sodium testosterone sulfate	QO_2	Per cent inhibition	Sodium androsterone sulfate	QO_2	Per cent inhibition
—	76.0	Control	—	86	Control
3.4×10^{-4}	45.0	41.0	9×10^{-5}	81.5	18.9
6.8×10^{-4}	32.0	58.0	1.3×10^{-4}	68.0	26.7
1.0×10^{-3}	13.8	81.5	1.8×10^{-4}	61.0	27.8
1.4×10^{-3}	4.0	95.0	2.3×10^{-4}	60	33.4
1.7×10^{-3}	5.0	94.0	2.7×10^{-4}	57.4	62.3
2.0×10^{-3}	2.6	96.0	3.2×10^{-4}	34.7	92
			3.6×10^{-4}	0	100
"Soluble" testosterone	QO_2	Per cent inhibition	diethylamino-ethanol · HCl	QO_2	Per cent inhibition
—	74	Control	—	87	Control
9×10^{-6}	66	10.8	3.5×10^{-5}	91	—
1.8×10^{-5}	36	51.5	3.5×10^{-5}	94	—
2.7×10^{-5}	14	81.0			
3.6×10^{-5}	8	89.0			
4.5×10^{-5}	3	96.0			
5.4×10^{-5}	2	97.5			

Preliminary results suggest that the water-soluble androgens also have an inhibitory effect on the lactic dehydrogenase system.

The curves of Figure 1 were derived from the data in Table 1 by plotting per cent inhibition of oxygen consumption by "soluble" testosterone for both malic and succinic dehydrogenase systems. Similar curves are obtained for inhibition by the sodium salt of testosterone sulfate of the malic dehydrogenase system and for the inhibition by sodium androsterone sulfate of the succinoxidase system. The inhibition of succinoxidase by sodium testosterone sulfate yields an atypical curve, no explanation for which is at hand.

Incubation time had no apparent effect on the inhibition of succinoxidase in liver homogenate by "soluble" testosterone in an experiment in which succinate was added from the side arms into the main chamber of the Warburg vessels at successive time intervals.

Figure 2 shows the effect of added homogenate on the testosterone inhibition of succinoxidase. Data are plotted according to Ackermann and Potter (1949). At each inhibitor concentration the same amount of enzyme is "bound" regardless of the amount of homogenate present.

DISCUSSION

The most interesting observation noted was the inhibition of the malic dehydrogenase system by "soluble" testosterone and by sodium testosterone sulfate. Erway *et al.* (1947) have reported that sodium androsterone

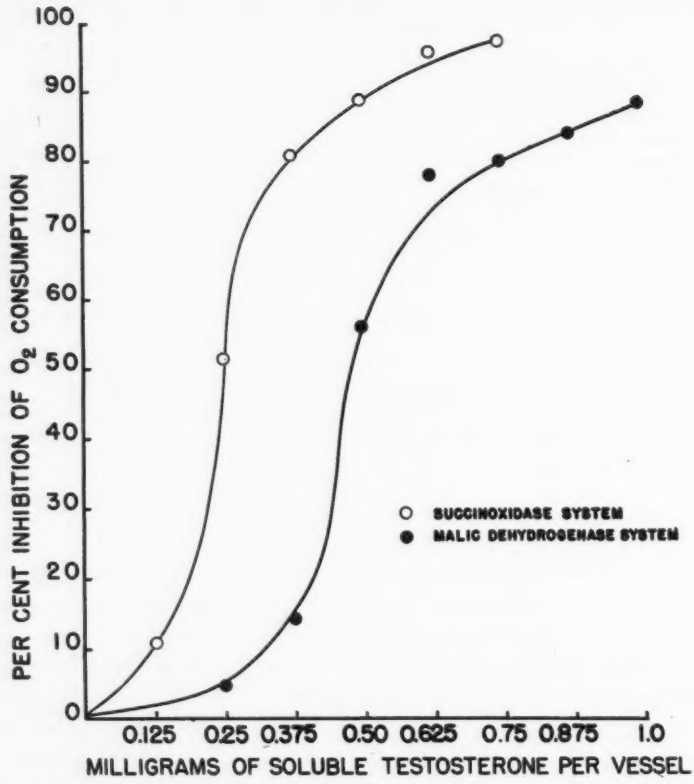

FIG. 1. The effect of concentration of testosterone 17-β diethylaminoethylcarbonate hydrochloride ("soluble" testosterone) on the malic and succinic dehydrogenase systems. 0.125 mg. "soluble" testosterone is equivalent to 9×10^{-6} molar concentration.

sulfate has no significant effect on this enzyme system. They used inhibitor concentrations of 10^{-4} molar.

The inhibition of the succinoxidase and malic dehydrogenase systems by water-soluble steroids used appeared not to fit classical formulations (Lineweaver and Burk 1932). However, the data are compatible with the "irreversible" type of inhibitor described by Ackermann and Potter (1949). A definite reduction in oxygen consumption is effected by a given increment of inhibitor and is not reversed by increasing the amount of enzyme present (Fig. 2). Bain (1949) has demonstrated one instance of this type of inhibition in the effect of certain phosphate esters (diisopropylfluorophosphate, tetraethylpyrophosphate) on cholinesterase. Hayano and Dorfman (1951) showed a similar relationship for the inhibition of a d-amino acid oxidase by desoxycorticosterone.

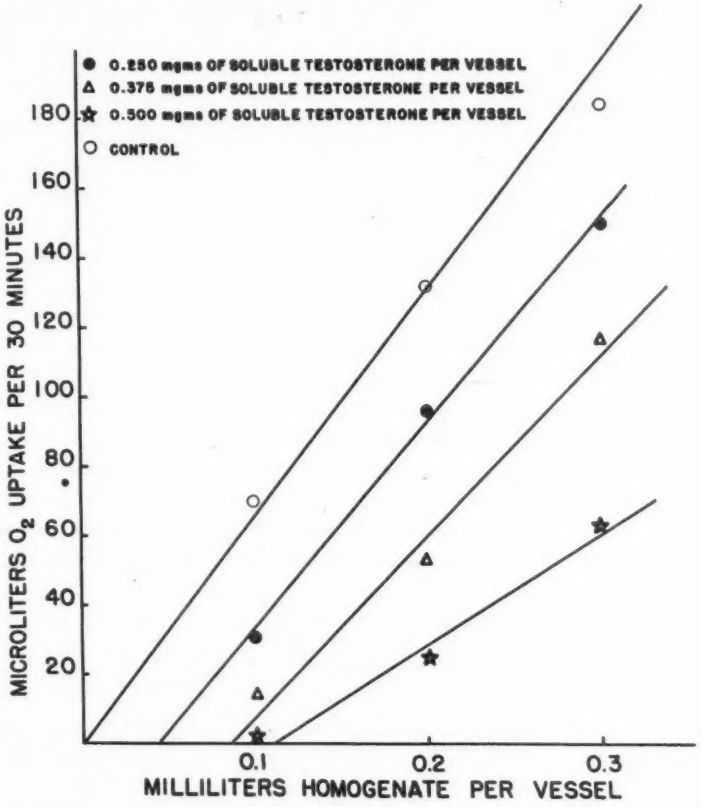

FIG. 2. The effect of increasing amounts of homogenate on the inhibition of the succinoxidase system by different amounts of testosterone 17-β diethylaminoethylcarbonate hydrochloride ("soluble" testosterone). 0.250 mg. soluble testosterone is equivalent to 1.8×10^{-5} molar concentration.

Dr. D. M. Greenberg suggested that the sigmoid shape of the curves relating per cent inhibition to inhibitor concentration (Fig. 1) resembles an adsorption isotherm. Such curves have been obtained, for example, for the amount of water vapor adsorbed on charcoal under conditions of varying pressure (Coolidge 1937). If we consider per cent inhibition analogous to inhibitor uptake, and inhibitor concentration analogous to "pressure," we may postulate that the steroid is adsorbed by the enzyme. The inhibition of saccharase by certain basic dyes has been described by Quastel and Yates (1936). When per cent inhibition was plotted against pH sigmoid curves similar to the ones plotted here were obtained. They suggested that the anion form of saccharase was better able to combine with basic dyes.

The significance of the variation in water solubility of the androgens mentioned above is not apparent. It may be that the long side chain of "soluble" testosterone makes this compound more susceptible to adsorption phenomena.

The site of action of the androgens on the dehydrogenase systems is thought to be the dehydrogenases themselves. Evidence for this has accumulated mainly by inference after other hydrogen carriers were bypassed or proved to be less susceptible to the inhibitors (Eisenberg *et al.*, 1949). A promising method of approach would seem to be fractionation of the components of the Warburg reaction mixtures in an attempt to recover the enzyme-inhibitor complex. Such an approach might also be used to study the enzyme-inhibitor "binding."

SUMMARY

The effects of water-soluble androgens on malic dehydrogenase and succinoxidase of rat liver homogenates were studied using conventional Warburg techniques. These substances were found to inhibit both enzyme systems in a manner suggesting an irreversible combination of enzyme and inhibitor. The data were also suggestive of some type of adsorption phenomenon involving enzyme and steroid.

Acknowledgements

The steroids used were kindly supplied by Dr. E. Oppenheimer of Ciba Pharmaceuticals.

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PATHWAYS OF ELIMINATION OF C^{14}-LABELED THYROXINE IN THE RAT[1]

H. M. KLITGAARD, H. J. LIPNER,[2] S. B. BARKER,[3] AND T. WINNICK

From the Department of Physiology and the Radiation Research Laboratory, State University of Iowa, College of Medicine, Iowa City, Iowa

INTRODUCTION

TAUROG, Briggs, and Chaikoff (1951) using I^{131}-L-thyroxine demonstrated the presence of an unknown metabolic product, in addition to thyroxine, in the bile of rats. These investigators (1952) then reported that the thyroxine-containing derivative may be a glucuronide, but not a peptide. They further concluded that the conjugate is the primary metabolic product of endogenous thyroxine, and that the bile is the chief pathway of elimination for this hormone.

The present study is concerned with the elimination of DL-thyroxine--1-C^{14} in the bile, urine, and expired air. Further analysis of the bile has indicated that the thyroxine conjugate is not a peptide.

METHODS

DL-thyroxine-1-C^{14} used was that synthesized by Wang, Hummel, and Winnick (1952).[4] Its specific activity was 5.3 μc per mg. In all radioactivity measurements, the observed counts per minute were converted into equivalent μgm. of thyroxine, by reference to the known specific activity of the administered C^{14}-thyroxine. Samples were generally counted to within a probable error of 2 or 3%, and the values corrected for self-absorption. By way of illustration, 0.1 ml. of bile had of the order of 300 counts per minute, or 20 times background, with our counter.

Adult male Sprague-Dawley rats, fed ad libitum, were employed. The bile duct was cannulated with polyethylene tubing (inside diameter, 0.011 inch). When a good rate of flow was observed, the abdomen was closed, and the animal suspended in a restraining harness. Thyroxine was injected subcutaneously at a dose level of 780 μgm. (one μM) per kilo body weight. Bile samples were collected in 2 ml. portions over a 12 to 14-hour

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[2] Baxter Laboratories Fellow.

[3] Present address, Department of Pharmacology, Medical College of Alabama, Birmingham 5.

[4] The C^{14} for the synthesis of the thyroxine was obtained on allocation from the U. S. Atomic Energy Commission.

period. The radioactivity of the whole bile was determined by drying 0.1 ml. aliquots on aluminum plates, and counted in a thin mica window Geiger counter.

Paper chromatograms were performed as described by Taurog and co-workers (1951). Aliquots of 0.1 ml. bile were chromatographed immediately after the bile was collected. After the chromatogram was completed, the paper was cut into 1 cm. strips, and each strip extracted three times with 0.5 ml. of 0.01N NaOH. The extracts were air dried on aluminum plates for counting. Approximately 53% of the added thyroxine activity was recovered from the paper chromatograms.

The whole bile was subjected to the Van Slyke ninhydrin-carbon dioxide method (1941) which indicates the presence of free carboxyl and amino groups in amino acids. These determinations were performed at pH 2.5 on 0.1 ml. bile samples. The $BaCO_3$ was collected and counted as described by Winnick (1950).

A Benedict closed circuit metabolic apparatus was employed for the collection of the expired $C^{14}O_2$. The latter was removed from the expired air by bubbling the circulating gases through a 1N NaOH solution which was replaced at 2 or 3-hour intervals. Aliquots of the NaOH solutions were treated with excess $BaCl_2$, the precipitated $BaCO_3$ washed, dried, and plated for counting.

The concentration of C^{14} activity in the urine was determined on 0.1 ml. aliquots of samples, obtained at the various time intervals. The urine, which was collected from rats placed in metabolic cages, was plated as described for the bile.

RESULTS AND DISCUSSION

Figure 1 shows that the C^{14} of carboxyl-labeled DL-thyroxine is most rapidly eliminated by way of the bile. About 20% of the radioactivity was found in the bile in 12 hours. Almost identical results were obtained in 5 repeated experiments. Taurog and co-workers (1951) give a comparable value of 28% eliminated in 12 hours, for a subcutaneous injection of 330 μgm. of I^{131}-labeled L-thyroxine per kilo.

The appearance of 10% of the C^{14} in respiratory carbon dioxide in 12 hours indicates that the alanine portion of the thyroxine molecule is catab-

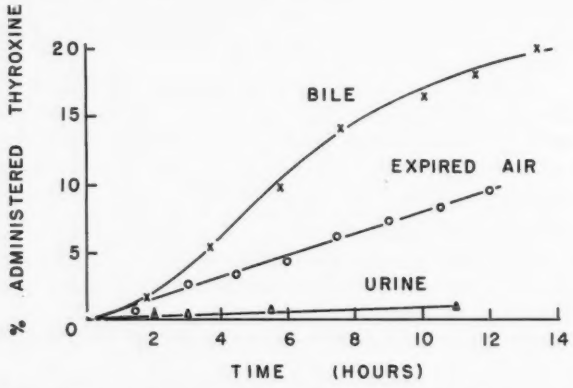

FIG. 1. Elimination of C^{14}-carboxyl labeled thyroxine by different routes.

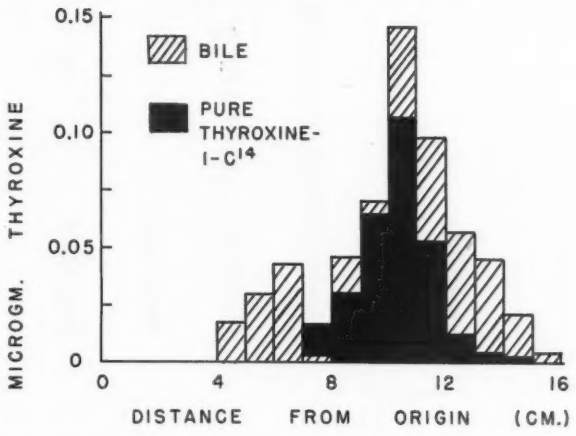

FIG. 2. Chromatogram of 0.1 ml. aliquot of a 2 ml. sample of bile, taken 1.8 to 3.8 hours after administering thyroxine-1-C^{14}.

olized. Essentially the same rate of elimination of $C^{14}O_2$ was obtained in 5 other experiments.

A relatively low C^{14} concentration, about 1% of the total dose, appeared in the urine in 12 hours. The average value was 1.2% for 10 such experiments. This radioactivity may represent, in part, urea derived from $C^{14}O_2$ by way of the ornithine cycle. Gross and Leblond (1949) reported a urinary excretion of about 10% of intravenously administered I^{131}-thyroxine in rats after 24 hours. Most of the I^{131} could not be extracted from the urine by butanol, and was referred to as "non-thyroxine" iodine.

The results of a paper chromatogram performed on bile from a rat given C^{14}-thyroxine is shown in Figure 2. The small peak accounted for 10%, and the larger, more rapidly moving peak 53%, of the total C^{14} in the bile sample. The position of the larger peak is identical with that of thyroxine. These results show that, at the 780 μgm./kilo dosage, free thyroxine predominates in the bile. This conclusion is in accord with the finding of Taurog and co-workers (1952) that large doses of thyroxine-I^{131} are conjugated to only a very limited extent in the rat. Because of the limitations imposed by the specific radioactivity of our thyroxine-1-C^{14}, it was not possible to employ the latter at physiological levels.

The data in Figure 3 indicate that virtually all of the C^{14} of the bile is released by ninhydrin treatment. The agreements are within the limits of experimental error. Van Slyke, Dillon, MacFadyen, and Hamilton (1941) have shown that amino acids are decarboxylated by ninhydrin only when both amino and carboxyl groups are in the uncombined state. Although

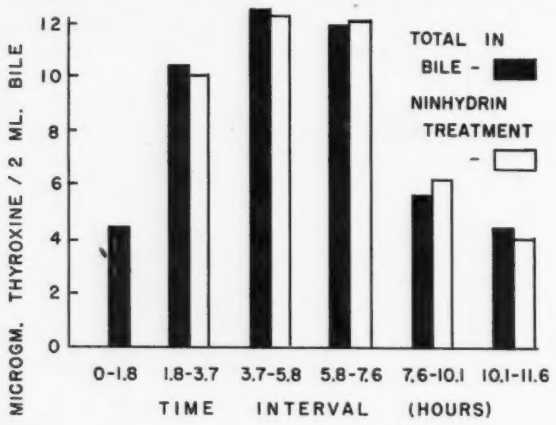

FIG. 3. Thyroxine excretion, as measured by total C^{14} in bile, and by $C^{14}O_2$ released from bile by ninhydrin.

chromatography indicates that a minor portion of the thyroxine exists in conjugated form, this fraction may be bound through the phenolic hydroxyl group, and hence would still react with ninhydrin.

SUMMARY

The various routes of elimination of thyroxine-1-C^{14} in normal adult rats were investigated. Labeled thyroxine was administered subcutaneously at a level of 780 μgm./kilo. The bile was the major route of excretion, and contained about 20% of the C^{14} after 12 hours. Approximately 10% of the C^{14} appeared in the expired CO_2, and 1% in the urine, in this period.

Paper chromatographic analysis of a bile sample taken between 2 and 4 hours, revealed the presence of a small secondary component, in addition to a large thyroxine peak. Treatment of bile samples with ninhydrin resulted in a quantitative release of C^{14} as $C^{14}O_2$. This finding indicates that both amino and carboxyl groups of the thyroxine in the bile are in an uncombined state.

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ESTIMATED DURATION OF THE SPONTANEOUS ACTIVATION WHICH CAUSES RELEASE OF OVULATING HORMONE FROM THE RAT HYPOPHYSIS[1]

JOHN W. EVERETT AND CHARLES H. SAWYER[2]

From the Department of Anatomy, Duke University School of Medicine, Durham, North Carolina

INTRODUCTION

NEUROGENIC activation of the rabbit adenohypophysis causing discharge of ovulating hormone is now considered to be accomplished within about one minute after coitus (Sawyer, Markee *et al.*, 1947, 1949). Actual discharge into the bloodstream of hormone sufficient for ovulation requires approximately one hour in that species (Fee and Parkes, 1929).

Whereas, in spontaneously ovulating animals, discharge of the hormone was long thought to be invoked by direct action of ovarian steroids on the hypophysis, there is now strong evidence that this effect is mediated by the hypothalamus (Sawyer, Everett and Markee, 1949; Everett and Sawyer, 1949). Activation of the hypophysis in cyclic rats occurs within a restricted period of time during the day of proestrus (Everett, Sawyer and Markee, 1949). In our colony this critical period is between 2 and 4 P.M. for the great majority of animals (4-day cycle), about 9 to 11 hours before ovulation. The injection of certain blocking agents at 2 P.M. or earlier will prevent ovulation, while their injection at 4 P.M. will not usually interfere with it.

Studies with the rabbit have demonstrated that antiadrenergic agents of the β-haloalkylamine series (Dibenamine and SKF-501) and certain anticholinergic agents (atropine and Banthine) will block the reflexogenous stimulus to the hypophysis when injected intravenously within a fraction of a minute after coitus (Sawyer, Markee *et al.*, 1947, 1949, 1950, 1951). These agents are similarly effective in blocking the "spontaneous" activation of the hypophysis in cyclic rats. In addition, the barbiturates have been effective in the latter species, apparently because they can be intro-

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[2] Present address: Department of Anatomy, University of California Medical Center, Los Angeles 24, California.

duced in advance of the known critical hour (Everett and Sawyer, 1950). Of the several agents, the most dependable and practical ones in rats have been atropine and Nembutal.

The purpose of the present investigation is to estimate more closely than heretofore the actual duration of the stimulus activating the rat hypophysis. Our information from work cited above simply defines a 2-hour period at some time during which hypophyseal stimulation takes place among the various 4-day cyclic rats of the colony, without indicating how long the stimulus lasts in any one individual. While it might conceivably be momentary as in rabbits, evidence to the contrary was encountered in study of progesterone-induced ovulation and its blockade by atropine (Everett and Sawyer, 1949). Among 47 rats receiving first progesterone and then atropine 2 to 6 hours later, 8 animals showed neither complete blockade nor complete activation. Their ovaries on the following morning characteristically contained follicles in various stages of preovulatory swelling, instead of growing follicles or full sets of fresh corpora lutea. Inasmuch as spontaneous activation of the hypophysis had always been fully blocked when injection of atropine was suitably timed, we could hardly explain these partial effects by failure of the drug itself. Rather, they appeared to represent interruption of activation which was in progress at the time of atropine injection. Their frequency indicated that activation of the rat hypophysis proceeds over a relatively long time. The experiments decribed below support such a conclusion.

MATERIALS AND METHODS

The data included here in the definitive series were derived from 113 adult female rats, including 64 from previously reported work (2 P.M. and 4 P.M. injections of atropine or Nembutal) and 49 new ones. In addition 25 such animals were used in 2 control series. All were members of our inbred Vanderbilt (Osborne-Mendel) strain. For increased uniformity it was required of each animal in both categories, not only that she have a history of at least two 4-day cycles in sequence immediately preceding the experimental cycle (cf. Everett, Sawyer and Markee, 1949, and Everett and Sawyer, 1950), but also that her vaginal smear on the morning of the expected current proestrus be entirely free of leucocytes. A few individuals in the previously reported 2 P.M. and 4 P.M. groups were thus excluded for the present purpose.

The 49 rats were divided into 5 groups of 9 or 10 each and the members of each group were injected during the afternoon of proestrus with the standard dose of atropine sulphate (700 mg./kg. in Ringer-Locke solution subcutaneously; cf. Sawyer, Everett and Markee, 1949) at one of the following approximate times: 2:40 (2:38), 2:50, 3:00, 3:15 and 3:30. The actual time for any one rat was rarely more than 2 minutes on either side of one of these mean values. Although an unknown interval must be assumed to follow injection, before the atropine reaches the site of blockade in adequate concentration, such delay was common to all cases. To keep this factor as uniform as possible, the same injection site was always used; equally divided amounts of the solution were placed in either groin.

On the following morning between 8 and 10 A.M. the animals were killed with illuminating gas and were immediately autopsied. The ampullae of the oviducts were searched for ova and the excised ovaries were examined for fresh rupture points. Unless full sets of tubal ova and rupture points were found, both ovaries were preserved in Zenker's fluid. Serial sections (10μ) were stained by a modified Mallory tri-acid technique.

Criteria of complete blockade, partial activation and complete activation were as follows. To indicate *complete blockade*, a given pair of ovaries must be free of any trace of luteinization, as shown by close scrutiny of every graafian follicle in the serial sections. No evidence of secretion of secondary liquor folliculi should be present. Every ovum must contain an intact ovocyte nucleus. The basal layer of the granulosa must be distinct throughout with its nuclei oriented perpendicular to the basement membrane. This produces a clear demarkation between granulosa and theca interna, readily seen even in oblique or tangential sections. At the other extreme, *complete activation* was said to have occurred if the ovaries contained fresh corpora lutea in stages comparable with those normally found at the given hours of autopsy on the morning after ovulation. No significance was attached to the finding of occasional unruptured, swollen or luteinized follicles, provided there were in addition 7 or more normally developed corpora lutea. Actually less than 7 normal corpora would have been accepted had the occasion arisen and had it been demonstrable that no follicle had failed to rupture. The least evidence accepted for *partial activation* was the presence of a localized area of luteinization (lutein plaque) in a small segment of a follicle wall. In such a region distinct orientation of the basal granulosa nuclei is lacking, the granulosa cells are somewhat enlarged, the basement membrane is indistinct or absent, the theca interna is thickened and its capillaries may actually have invaded the granulosa. The remainder of the follicle may be quite unaffected. In 3 cases such lutein plaques in 2 to 4 follicles were the only indication of activation, while in others one or more plaques coexisted with additional follicles in outright preovulatory swelling. The most extreme effect short of complete activation was the presence in one pair of ovaries of 7 very freshly ruptured follicles, together with 5 unruptured follicles in late swelling. (Various examples of submaximally stimulated follicles are photographically illustrated in a recent review which incorporated a preliminary account of the present experiments—Everett, 1952).

RESULTS

Figure 1 graphically illustrates the results of attempted blockade according to time of injection. The 2 P.M. and 4 P.M. values comprise pooled data from previous atropine and Nembutal experiments (Everett, Sawyer and Markee, 1949; Everett and Sawyer, 1950), excluding a few animals whose vaginal smears had contained a few leucocytes on the morning of presumptive proestrus. Dibenamine data were not acceptable because of the observed failure or partial failure of blockade in a number of animals under optimal conditions of timing.

Inspection of Figure 1 shows that from practically complete blockade by injection of atropine (or Nembutal) at 2 P.M. there was a progressive increase in the proportionate numbers of rats showing activation, until from 3:30 P.M. onward nearly all had been fully activated before injection. For the group as a whole, the frequences of cases in which activation had begun at the various injection times appeared to follow curvilinear re-

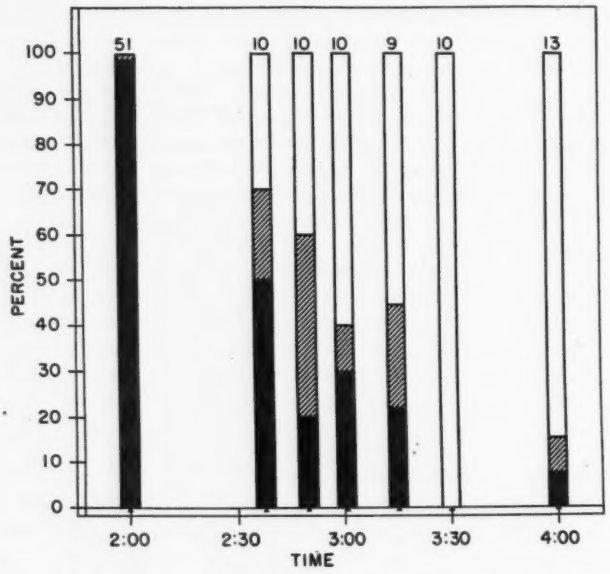

FIG. 1. Results of injection of atropine on the day of proestrus at different times between 2:00 and 4:00 P.M. Solid black: complete blockade. Cross-hatching: partial blockade. White: complete ovulation. The number above each bar indicates the respective number of rats in that time-group. The 2:00 and 4:00 P.M. data are from previous work with atropine and Nembutal (From Everett, 1952, by courtesy of the Ciba Foundation).

gression, for the following considerations. During the first 50 minutes 65% of 20 rats had begun activation, while during the succeeding 40 minutes the percentage increased to only 83% of the remaining 29 rats. Even as late as 4 P.M. 1 case of complete blockade and 1 of partial blockade were encountered. While the former may have been a rare animal which whould have failed to ovulate without atropine, the latter was not. In her case activation must have begun only a short time before injection. It seems probable that an average of the combined 3:30 and 4 P.M. data gives a more nearly true value than either alone.

The occurrence of 9 cases of partial activation, rather uniformly distributed among the 2:40–3:15 time-groups, confirms the earlier impression that the activation stimulus is not momentary. Such a conclusion, however, requires that under the conditions of administration of atropine in massive doses by the subcutaneous route the drug abruptly assumes full blocking concentration at its locus of action, with no significantly prolonged interval during which it would only moderately diminish the

strength of stimulus. The following control experiments seem to give this assurance.

A threshold dose for subcutaneous injection was arrived at empirically: 230 mg./kg. (23 mg. atropine sulphate per ml. of physiological saline). This amount was administered to each of 15 proestrous 4-day cyclic rats at 2:00 P.M. At autopsy next morning the tubes were searched for ova and the excised ovaries were critically examined under the dissecting microscope. In the absence of tubal ova all follicles showing any degree of hyperemia were dissected out with iridectomy scissors and were individually crushed in a small drop of saline between slide and coverslip for microscopic examination. Eight of the 15 rats were judged to have been completely blocked, while 6 rats had ovulated completely. In the remaining animal 3 follicles were markedly hyperemic and when these were crushed prominent corona radiata were seen. Maturation of the ova was in progress and the first polar body was seen in one instance. Partial activation in this one exceptional animal may be assigned to an unusually early beginning of hypophyseal activation, similar to the one case from the Nembutal series included in the 2:00 P.M. group in figure 1. The absence of *numerous* examples of partial activation in the present series, intergrading between complete blockade and complete ovulation, attests an essentially all-or-none effect of atropine at threshold level and signifies that no prolonged interval of partial effectiveness of atropine precedes the time at which full blockade is accomplished.

The second series of control experiments approached the question in another way. If the cases of partial blockade in the definitive series (Fig. 1) were mainly representative of a prolonged interval of partial effectiveness of atropine associated with slow absorption from the subcutaneous site, the intravenous injection of the maximum amount tolerated, immediately before the standard subcutaneous dose at some time during the critical period, should have an all-or-none effect. Most female rats of this strain will tolerate intravenous injection of 70 mg./kg. of atropine sulphate (35 mg./ml. in Ringer-Locke solution). This amount was now given to each of 10 proestrous 4-day cyclic rats at one or another time between 2:30 and 3:20 P.M., the entire amount being introduced within 70 to 80 seconds. (Two additional rats died within a few minutes after injection.) Subcutaneous injection of the 700 mg./kg. dose was completed within a minute afterward. Marked tremors were noted before the intravenous injections were complete and involuntary contractions of various muscle groups were regularly seen when the animal was released. It is significant that in 3 of the 10 rats partial blockade was encountered. At autopsy on the morning after injection, 2 of these had a single follicle showing corona formation and maturation changes, while the third animal had shed 2

ova and most of her unruptured follicles were markedly hyperemic. This considerable number of animals showing partial effects supports the interpretation that they arise from abrupt interruption of a lengthy process of hypophyseal activation.

Inasmuch as the chance of interrupting a progressive process is a direct function of its duration, the latter can be estimated in the present instance from the percentage partially activated. If regression of percentage blocked were linear with respect to time, a direct transformation could be made from percentage partially activated to minutes elapsed. Inasmuch

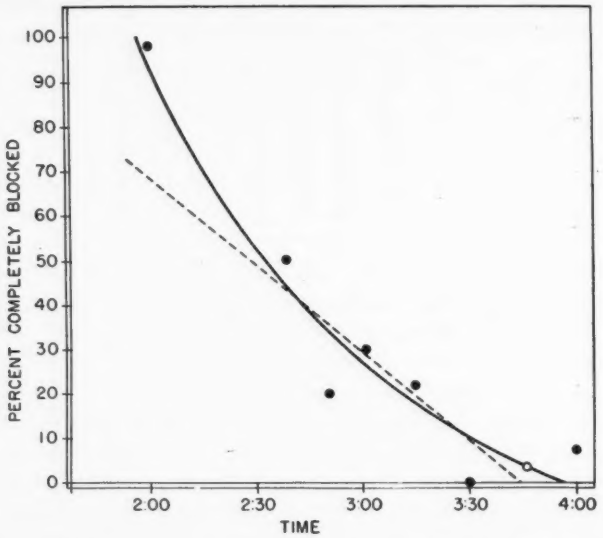

FIG. 2. Diminishing frequency of complete blockade after 2:00 P.M., inversely measuring the beginning of hypophyseal activation. The logarithmic curve is mathematically fitted to the percentages represented by solid circles (the open circle represents the average of the 3:30 and 4:00 P.M. values). The straight (broken) line is approximated to the curve between the 2:40 and 3:30 points (see text).

as the regression is curvilinear, however, certain approximations are required to allow such transformation.

In Figure 2 a logarithmic curve is mathematically fitted to the points representing percentages completely blocked. A straight (broken) line is approximated to the logarithmic curve between the 2:40 and 3:30 points. The slope of this line is such that 10% on the y axis corresponds to 15.3 minutes on the x axis. In terms of this slope, the 9 cases (18.4%) of partial activation among 49 rats of the 2:40–3:30 groups correspond to 28 minutes on the time axis.

This value, however, is admittedly a very rough approximation and has little significance in itself. An important source of error is chance variation in the percentage partially activated. By use of Fisher's table $VIII_1$ (Fisher and Yates, 1949), entered with $a = 9$ ($a/N = 9/49 = 0.184$) the lower limits of expectation were obtained.[3] At probability levels of 0.025 and 0.005 these limits were 10% and 8%, respectively, corresponding to 13 and 10 minutes (fig. 3). Thus, the probability is high (0.975) that duration of stimulation is greater than 13 minutes, based on the slope of the straight line from figure 2. An extremely unlikely lower limit may be calculated

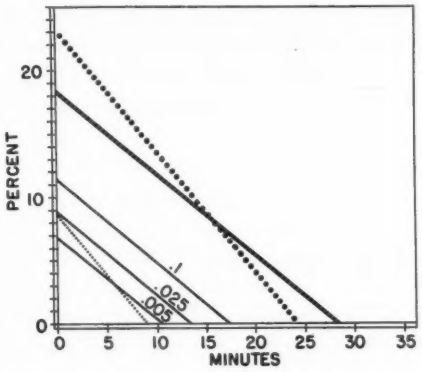

FIG. 3. Relation between percentage partially blocked and time. The heavy solid line has the same slope as the broken line in figure 2. Lower limits of expectation at three levels of probability are represented by the narrow solid lines. Significance of the dotted lines is given in the text.

by disregarding the evident curvilinearity of regression and employing as the steepest plausible slope a straight line drawn between the 2:40 P.M. and 3:30 P.M. values. Since on this assumption all activation would be completed before 3:30, this group is excluded in calculating the percentage partially activated and Fisher's table $VIII_1$ is entered with $a = 9$ ($a/N = 9/39 = 0.231$). At the probability level of 0.005 this gives a lower limit of 9 minutes (Fig. 3, lower dotted line).

The calculated limits do not take into consideration the numerous cases of partial activation in other comparable experiments, e.g. the progesterone-atropine series cited earlier (Everett and Sawyer, 1949). Among 78 appropriate examples from such experiments, 20 (24%) were classed as partial activation. Thus the frequency of partial activation in the present series appears to be more truly representative than the cal-

[3] The upper limit was given directly by the fact that 3 of 10 rats in the 2:40 group were fully activated. Their periods of activation must have been less than 40 minutes.

culations imply. Consequently it seems reasonable to judge that in any given rat the duration of stimulus is within the range of 20 to 35 minutes.

DISCUSSION

The results confirm the earlier conclusion (Everett, Sawyer and Markee, 1949; Everett and Sawyer, 1950) that in 4-day cyclic rats of our colony a specific neurogenic stimulus, essential to the discharge of ovulating hormone, passes to the adenohypophysis within restricted, predictable time limits on the afternoon of the day of proestrus. In addition, it is now shown that this stimulus in any one individual is considerably shorter than the 2-hour critical period (2 to 4 P.M.) previously defined. On the other hand, the stimulus lasts much longer than the reflexogenous one in the rabbit. In that species the cholinergic (atropine-sensitive) phase is thought to be completed early in the first minute post-coitum, while the adrenergic (Dibenamine-sensitive) phase is only slightly longer, approximately 1 minute (Sawyer, Markee and Hollinshead, 1947; Sawyer, Markee and Townsend, 1949; Sawyer, Markee and Everett, 1950). In the rat the cholinergic phase of the stimulation process lasts for over 9 minutes, more probably between 20 and 35 minutes and certainly less than 40 minutes.

It seems likely that in the rat the central (Nembutal-sensitive) phase and the cholinergic and adrenergic phases of the stimulation process take place almost concurrently. Time differences of the order found in rabbits would be obscured by the far greater over-all duration.

A quantitative relationship apparently exists between length of time of hypophyseal stimulation and the amount of ovulating hormone released. Such an interpretation rests on the finding in rabbits that none of the blocking agents interferes directly with passage of ovulating hormone from the hypophyseal cells into circulation (Sawyer, *et al.*, loc. cit.). Since a species difference in this respect is unlikely, the numerous cases of partial blockade in the present series should then represent incomplete release resulting from foreshortened stimulation. Indeed, if the normal period of stimulation approximates half an hour, stimulation and release may proceed concurrently. While in rabbits about an hour is required for release (Fee and Parkes, 1929), the time in rats could easily be somewhat shorter.

It should be noted that in at least two of the species in which ovulation depends on copulation, relatively prolonged stimulation from lengthy or repeated copulation is indicated (*ferret*, Hill and Parkes, 1932; *mole shrew*, Pearson, 1944). In the mole shrew 1 to 3 copulations per day are said to be ineffective, whereas ovulation regularly follows when the number is increased to 10 to 15 for another two days. In contrasting the rabbit and the ferret, Hill and Parkes spoke of the "trigger" mechanism characteristic of the former species, although its momentary nature had not been eluci-

dated at that time. It seems probable that in spontaneous ovulators and reflex ovulators alike there may be rather wide species variation in the duration of the stimulation process.

There are some indications that under normal circumstances the rat hypophysis continues to be stimulated for a considerably longer period than that which is just sufficient to release the minimal amount of gonadotrophin capable of ovulating all the follicles. Everett, Sawyer and Markee (1949) noted in the original 4 P.M.-atropine series that while all animals ovulated, the corpora lutea from the preceding cycle failed to store visible cholesterol within the usual time. Such cholesterol storage has been shown to be a function of LH (Everett, 1947). Another function of LH is seen in the depletion of cholesterol in the interstitial tissue (Claesson and Hillarp, 1947; Everett, 1949). In our 4-day cyclic rats such depletion is normally so extreme on the morning after ovulation that the ovarian interstitium presents a dull, watery appearance. In the present experiments this extreme depletion was noted in only 24 of 35 rats (69%) in the "fully ovulated" category. It seems likely, therefore, that the estimate of 20 to 35 minutes simply represents the minimal stimulation necessary for ovulation and that the normal stimulation lasts for some longer time, causing relase of an excess of gonadotrophin. This factor of insurance may be reflected not only in the lipid changes in corpora lutea and interstitial tissue, but also in the uniformity of ovulation time observed in these animals.

SUMMARY

Previous study of 4-day cyclic rats (inbred Vanderbilt strain) has defined a 2-hour interval during proestrus, within which neurogenic ovulatory activation of the adenohypophysis takes place. In the present study, 49 such rats were injected with the standard blocking dose of atropine at different times during that interval and were autopsied the next morning. From the results, in particular from the relative frequency of partial blockåde, it is estimated that to release sufficient gonadotrophin for complete ovulation the required duration of stimulus is between 20 and 35 minutes. Collateral evidence suggests that the stimulus may normally continue somewhat longer, serving to release an excess of ovulating hormone.

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ORGANIC PHOSPHORUS COMPOUNDS IN THE TESTIS OF THE RAT AT VARIOUS AGES[1,2]

WILLIAM B. McENERY[3] AND WARREN O. NELSON

From the Department of Anatomy, College of Medicine, State University of Iowa, Iowa City, Iowa

NUMEROUS investigations have been made of the pentose nucleic acid (PNA) and desoxypentose nucleic acid (DNA) concentrations of various tissues and organs in different experimental animals. These have been summarized in tabular form by Davidson (1947, 1950), Schneider (1946) and Schneider and Klug (1946).

Cytochemical studies covering the content and distribution of nucleic acids in many tissues and organs have been reviewed by Davidson (1950), Brachet (1950) and Caspersson (1950).

Examination of the literature has shown that little attention has been given to the concentration and distribution of these important substances in the testis. McCarter and Stelges (1948) in an examination of methods for the determination of phosphorus-containing constituents in rat tissues have included a limited survey of organic phosphorus compounds in the testis. Using cytochemical techniques Brachet (1933, 1940, 1941) has studied insect and amphibian testes and Caspersson (1933, 1936) grasshopper testes.

The paucity of information concerning the nucleic acids of the testis, particularly the mammalian testis, has suggested the desirability of such a study in the rat by chemical and cytochemical techniques. In order to provide a more complete survey of other phosphorus-containing substances additional studies were made.

Acid-soluble phosphorus (ASP) which according to Lipman (1941) and

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[2] This paper is a part of the content of a dissertation submitted by the senior author in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Anatomy of the Graduate College of the State University of Iowa, February 1952.

[3] Present address: Department of Anatomy, School of Medicine, The Creighton University, Omaha, Nebraska.

Kalcar (1941) represents primarily intermediates and enzymes in carbohydrate and energy metabolism, was determined because of the reputed relationships between these and nucleic acid metabolism (Hevesy, 1948; Davidson, 1950). The lipid phosphorus (LP) fraction was studied since Claude (1939, 1940, 1941, 1949) has demonstrated a relationship between cytoplasmic pentose nucleic acid and phospholipid.

The present report, therefore, includes chemical data on the four phosphorus fractions and cytochemical data on the nucleic acids in the rat testis at different ages. Similar data for testes with experimentally induced impairments in spermatogenesis are being reported in a separate paper (McEnery and Nelson, 1952).

MATERIALS AND METHODS

Rats of the Long-Evans strain raised in this laboratory were used throughout the study. Animals were sacrificed when ten, twenty, thirty, forty, sixty and one hundred eighty days of age and the testes removed for chemical analysis and for cytochemical and routine histological preparations.

Chemical Determinations

Pentose nucleic acid phosphorus (PNAP), desoxypentose nucleic acid phosphorus (DNAP), total nucleic acid phosphorus (TNAP), acid soluble phosphorus (ASP) and lipid phosphorus (LP) were determined according to the method of Schmidt and Thannhauser (1945) as modified by Schneider (1946). In the case of each procedure duplicate determinations were made on testicular tissue from each animal except in the ten day series where the two testes from each of five animals were pooled. All were wet weight determinations and moisture contents were checked in each group.

Cytochemical Preparations

Portions of the testes were fixed in Zenker acetic fluid for one hour and fifteen minutes and prepared according to the Feulgen technique (Feulgen and Rossenbeck, 1924) for DNA and the methyl green pyronin technique for PNA and DNA (Brachet 1940, 1941). Since the latter technique is extremely capricious and for specificity should be accompanied by ribonuclease digestion of control sections the cytoplasmic basophilia demonstrated by it was used only as a rough index of PNA. It may be mentioned, however, that Taft (1951) in a recent paper offers substantial evidence for the specificity of the methyl green pyronin reaction as an indicator of the presence of both PNA and DNA.

Cytological Preparations

Portions of the testes were fixed in Bouin's fluid and stained with hematoxylin and eosin, Masson's and Mallory-Azan procedures.

RESULTS

Chemical Determinations

Table 1 presents the mean values for the concentrations of phosphorus in the PNA, DNA, TNA, ASP and LP fractions, and the calculated amounts of PNA and DNA along with standard errors and p values of the

differences between the means of the values obtained for these fractions in the animals of the younger age groups as compared with the equivalent values in adult animals. The corresponding absolute values (calculated amounts in both testes) are given in Table 2.

It is readily seen that as the age of the animals progresses toward maturity the testicular concentration of each of the phosphorus fractions

TABLE 1. THE MEAN CONCENTRATIONS OF THE FIVE P FRACTIONS AND THE CALCULATED MEAN CONCENTRATION OF PNA AND DNA FOR EACH AGE EXPRESSED AS MG. PER 100 GM. OF FRESH TISSUE

Group	No. of rats	PNAP	DNAP	TNAP	ASP	LP	PNA	DNA
10 day	24	120.01 ±6.459* 0.001†	25.81 ±0.964 0.001	141.32 +2.632 0.001	148.79 ±2.454 0.001	34.13 ±1.718 0.20	1272.10	260.68
20 day	22	110.84 ±0.417 0.001	26.67 ±0.842 0.001	137.85 ±2.213 0.001	126.98 ±1.707 0.001	37.39 ±0.861 0.01	1174.90	269.36
30 day	21	86.63 ±1.546 0.001	20.83 ±0.562 0.001	107.35 ±1.765 0.001	94.72 ±2.038 0.001	32.70 ±2.071 0.20	918.27	210.38
40 day	22	70.31 ±1.997 0.001	13.86 ±0.521 0.001	86.60 ±1.675 0.001	73.55 ±2.115 0.001	33.76 ±1.191 0.20	748.28	139.98
60 day	20	59.45 ±1.794 0.20	12.21 ±2.552 0.20	73.94 ±1.862 0.20	67.67 ±2.119 0.20	34.76 ±1.113 0.20	630.17	123.32
Normal Adult (180 day)	27	60.32 ±0.639	12.30 ±0.215	75.00 ±1.002	65.33 ±1.023	32.72 ±1.065	639.39	124.23

* Standard error of the mean.

† p values (from students table of t, Fischer and Yates 1947) for the differences between the mean values of the different age groups and the mean values of the normal adult group.

decreases with the exception of the lipid fraction which remains approximately constant (Fig. 1). Conversely in terms of absolute amounts there is a progressive increase in each of the phosphorus fractions with increase in age (Fig. 2).[4]

Total phosphorus determinations were made on five samples from normal adult testes and the mean values of these were compared with the sums of the mean values for the four fractions in the same testes. The total phosphorus content was greater by 21.73 mg. per 100 gm. and this difference presumably represents phosphoprotein (Schmidt and Thannhauser, 1945; McCarter and Stelges, 1948) and, of course, experimental error.

[4] Values for individual analyses and numerous illustrations may be found in the Ph.D. dissertation of the senior author (McEnery, 1952).

TABLE 2. THE CALCULATED MEAN VALUES OF THE FIVE P FRACTIONS AND THE CALCULATED MEAN VALUES FOR PNA AND DNA FOR EACH AGE EXPRESSED AS MG. IN BOTH TESTES

Group	No. of rats	Wt. of testes in gm.	PNAP	DNAP	TNAP	ASP	LP	PNA	DNA
10 day	24	0.041	0.049 ±0.002* 0.001†	0.010 ±0.0004 0.001	0.058 ±0.002 0.001	0.061 ±0.002 0.001	0.014 ±0.0006 0.001	0.527	0.108
20 day	22	0.209	0.231 ±0.006 0.001	0.055 ±0.001 0.001	0.0288 ±0.010 0.001	0.265 ±0.008 0.001	0.078 ±0.002 0.001	2.453	0.562
30 days	21	0.578	0.501 ±0.014 0.001	0.120 ±0.003 0.001	0.623 ±0.014 0.001	0.544 ±0.013 0.001	0.193 ±0.006 0.001	5.318	1.214
40 day	22	1.724	1.214 ±0.045 0.001	0.240 ±0.010 0.001	1.432 ±0.037 0.001	1.275 ±0.041 0.001	0.585 ±0.023 0.001	12.873	2.432
60 day	20	2.520	1.475 ±0.056 0.001	0.304 ±0.012 0.010	1.838 ±0.068 0.001	1.568 ±0.006 0.001	0.864 ±0.036 0.010	15.641	3.079
Normal Adult (180 day)	27	3.100	1.901 −0.042	0.387 −0.009	2.366 −0.052	2.053 −0.043	1.024 −0.029	20.155	3.913

* Standard error of the mean.

† p values (from students table of t, Fischer and Yates 1947) for the differences between the mean values of the different age groups and the mean values of the normal adult group.

Cytological and Cytochemical Observations

Normal Adult (One hundred and eighty days). The histologic and cytologic pictures are well known and need not be detailed here. Reference to them will be made in the course of the discussion since the changes in some of the phosphorus fractions are explained best in terms of changes in the cellular population of the testis.

The Feulgen preparations showed that the nuclei of the fibroblasts, endothelial cells and mast cells which are present in the intertubular areas are rich in fine granules of DNA while those of the Leydig cells contain only a few coarse granules. The sperm heads are Feulgen-positive throughout, and the nuclei of spermatocytes and spermatogonia give a strong positive reaction. The nuclei of the spermatids are strikingly low in DNA and the same is true for the Sertoli cell nuclei.

The methyl green pyronin preparations indicate the presence of considerable basophilia in the cytoplasm of the Leydig cells and much smaller amounts in other intertubular cells. All of the tubular cells possess cytoplasmic basophilia, the amount being proportional to the amount of cytoplasm in the cells. The Sertoli cells and spermatogonia appear to contain a little more than the others, and in the area where sperm maturation is

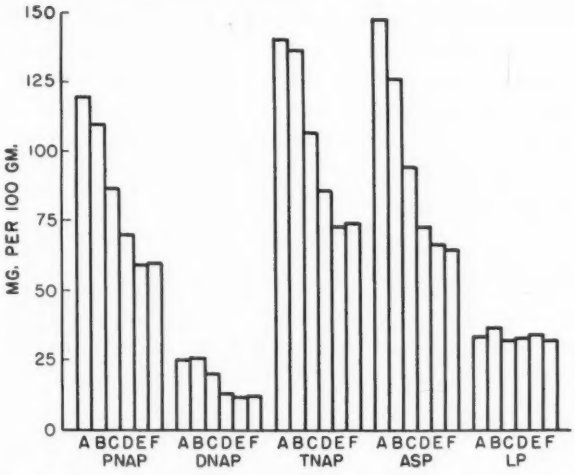

FIG. 1. Graph of the mean concentrations of the five P fractions for each age.

A = 10-day C = 30-day E = 60-day
B = 20-day D = 40-day F = 180-day

PNAP = Pentose nucleic acid phosphorus
DNAP = Desoxypentose nucleic acid phosphorus
TNAP = Total nucleic acid phosphorus
ASP = Acid soluble phosphorus
LP = Lipid phosphorus

taking place there are numerous extracellular, red, basophilic droplets. These droplets are not seen in all tubules but apparently coincide with a phase in the spermatogenic wave.

Ten-Day-Old Series. The testis of a ten-day-old rat contains occasional intertubular elements, which appear to be somewhat differentiated in the direction of Leydig cells. However, most of the cells in these areas are various types of connective tissue elements. The tubules contain Sertoli cells, actively dividing spermatogonia, and possibly a few young spermatocytes.

The intertubular cell nuclei give a Feulgen reaction comparable to that seen in the cells of these areas in the adult testis. Much the same is true of the nuclei of the tubular cells; indeed, the reaction for the Sertoli cells is somewhat more marked than in the Sertoli cells of the adult testis.

Those cells in the intertubular areas which appear somewhat differentiated are high in cytoplasmic basophilia while the mesenchymal cells give a weak reaction since they contain very little cytoplasm. In all tubular cells the cytoplasm is strongly basophilic.

Twenty-Day-Old Series. There are no Leydig cells in the testis of a twenty-day-old rat, the intertubular areas being made up of connective

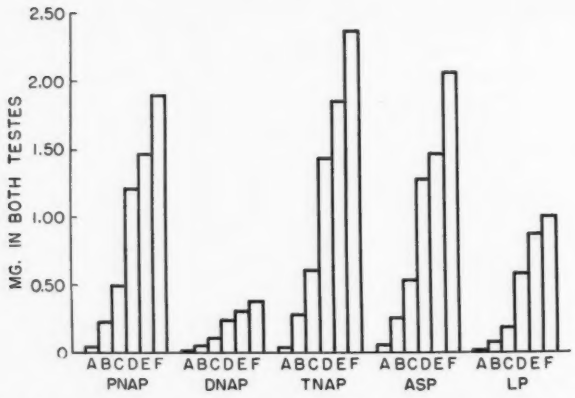

FIG. 2. Graph of the mean absolute values of the five P fractions for each age.

A = 10-day C = 30-day E = 60-day
B = 20-day D = 40-day F = 180-day

PNAP = Pentose nucleic acid phosphorus
DNAP = Desoxypentose nucleic acid phosphorus
TNAP = Total nucleic acid phosphorus
ASP = Acid soluble phosphorus
LP = Lipid phosphorus

tissue with a few mast cells, capillaries and other small blood vessels. The tubules contain Sertoli cells along with spermatogonia and spermatocytes.

The nuclei of all intertubular cells give a positive Feulgen reaction of about the same intensity as in similar cells of the adult testis. The Sertoli cell nuclei show a characteristic light diffuse pattern and the spermatogonia and spermatocyte nuclei both are rich in Feulgen positive material.

As in the ten-day-old testis the mesenchymal elements have little cytoplasm and show very little basophilia. The cytoplasm of the Sertoli cells, spermatogonia and spermatocytes is more basophilic than that of like cells in the adult testis.

Thirty-Day-Old Series. The testis of a thirty-day-old rat contains a few Leydig cells and the tubules have Sertoli cells, spermatogonia, spermatocytes and some spermatids.

The nuclei of the cellular elements of both the tubular cells and the intertubular areas give a Feulgen reaction similar to that seen in the normal adult. The Leydig cells have only a small amount of Feulgen-positive material and the spermatids give a weak reaction.

In methyl green pyronin preparations the Leydig cells show cytoplasmic basophila as do the Sertoli cells, spermatogonia, spermatocytes and spermatids. The Sertoli cells and spermatogonia appear to stain a little more deeply than the spermatocytes and spermatids.

Forty-Day-Old Series. With the appearance of some sperm the forty-day-old testis assumes adult characteristics and the Feulgen picture is almost identical with that seen in normal adult testes. The amount of cytoplasmic basophilia in the testes of animals of this series appears to be in general a little greater than that present in adult testes.

Sixty-Day-Old Series. The testes of sixty-day-old rats are identical cytologically and cytochemically with those of normal adult rats.

DISCUSSION

The observations on the normal adult rat testis which have been made in this investigation confirm, in most respects, such information as is available. Chemical data for all fractions are essentially in agreement with those reported by McCarter and Stelges (1948) with the exception of the LP fraction which they found to be 59 mg. per 100 gm. of tissue as compared with our value of 32.72 mg. per 100 gm. of tissue. The discrepancy may be due in part at least to the fact that we did not use the extensive extraction procedure which they employed. However, in our experience and in that of Schneider (1946), such a laborious fractionation did not materially increase the yield of LP. It is quite possible that on the basis of a limited number of analyses such as they carried out an inconsistently high value could easily be obtained.

The cytochemical observations on the normal adult testis which have been made in this study are entirely consistent with those reported previously. Brachet (1933, 1940, 1941) has shown that in insects and amphibians the spermatogonia contain more pentose nucleic acid than the other germ cells. In our preparations we noted more cytoplasmic basophilia in the peripheral cells of the tubules; however, it was somewhat difficult to decide whether or not this greater amount was due entirely to spermatogonia or whether the Sertoli cells were also involved. Caspersson (1933, 1936) working with grasshopper testes has shown that the amount of PNA is greatly increased in the older spermatocytes. It was our experience that the spermatocytic as well as the spermatogonial nuclei give a strong Feulgen positive reaction. This is in agreement with the theses of Brachet and Caspersson that the synthesis of DNA occurs at this time and that it is dependent on the amount of PNA present in spermatogonia and primary spermatocytes. We also found that the spermatid nuclei stained very lightly with the Feulgen reagent, an observation which is easily reconciled when we consider that the spermatid nuclei contain a haploid number of chromosomes. The occurrence of large basophilic droplets in the region of sperm maturation is particularly striking in view of evidence presented by McEnery and Nelson (1950, 1951) that there is accumulation of sudanophilic and Schiff positive lipid in the same distribution. Two possible

explanations may be offered for this finding. Lipoprotein complexes in the blood are well known (Troensgaard and Koudahl, 1926; Machebeouf, 1929; Machebeouf and Sandar, 1932; and Turner and Gibson, 1932) and it would seem possible that such a complex arising as a nutritive substance from the Sertoli cells, whose function is believed to be sustentacular and nutritive (Elftman, 1950; Nelson, 1951), would be visualized by lipid procedures as well as those for nucleoprotein. The second and possibly more reasonable explanation, in view of the fact that the appearance of these droplets coincides with a phase in the spermiogenic wave (Roosen-Runge, 1950, 1951), is that they arise from the sloughed cytoplasm of the spermatid during its metamorphosis. The latter explanation would be in good agreement with the observations of Claude (1939, 1940, 1941, 1949) that pentose nucleic acid exists in the cytoplasm in combination with phospholipid. Thus it is possible that the remnants of such a pentose nucleic acid-phospholipid complex are released when the spermatid is transformed into a spermatozoon and are visualized by the respective cytochemical procedures.

During the attainment of the adult condition changes in the different phosphorus fractions follow rather uniform patterns and the cytochemical observations are in good agreement with the patterns for the concentration of the nucleic acids. The concentrations of all fractions, with the exception of LP which remains relatively constant, decrease progressively as the age of the testis increases (Fig. 1), while the absolute values (amounts in both testes) for all of the phosphorus fractions increase progressively with the age of the testis (Fig. 2).

Adequate interpretation of these findings requires that we examine the morphologic changes which take place in the testis between ten and one hundred eighty days of age. In the first place there is approximately an eighty fold increase in weight. This is brought about by increases in the size of cells and by increases in their number. Secondly, the attainment of maturity involves the appearance of new cell types. The former, as we shall point out, appears to exert the major influence on the absolute values of the phosphorus fractions while the latter exerts more influence on the variations in concentration.

Two points must be developed in order to clarify this. In the first place it is well known that PNA is found chiefly in the cytoplasm. This is apparently also the case for the ASP fraction. Therefore tissue made up of cells having a large amount of cytoplasm (low nuclear-cytoplasmic ratio) will be high in those two substances whereas tissue made up of cells having little cytoplasm (high nuclear-cytoplasmic ratio) will have low concentrations of these substances. DNA on the other hand is for the most part (probably exclusively) in the nucleus and is actually a very important constituent of the chromosomes. Thus the DNA content is affected by the

nuclear cytoplasmic ratio of cells making up a given tissue. Furthermore, in the case of the testis there are cells with haploid as well as cells with diploid numbers of chromosomes. This will also influence the content of DNA. In fact, Boivin, Vendrely and Vendrely (1948) found that the nuclei of somatic cells from animals of a given species have twice as much DNA as the corresponding sperm (haploid) nuclei. Thus in the adult testis the concentrations of the various phosphorus fractions will be a function of the numbers of the various cell types present at that time.

In the second place attention must be directed to the cytologic picture of the testis at each age included in this study. At ten and twenty days of age the seminiferous tubules of the rat testis contain only cells which have considerable cytoplasm, namely Sertoli cells, spermatogonia and primary spermatocytes, while at thirty days secondary spermatocytes and spermatids have made their appearance and at forty days sperm are in evidence. After forty days no new cell types are added and further changes involve only increases in the numbers of cells, particularly those of the more mature types. Three significant generalizations can therefore be made with regard to the growth and maturation of the testis. First, as new cell types are added the total nuclear-cytoplasmic ratio of the cells of the testis changes from a lower to a higher value. Secondly, the last three cell types to appear, namely, the secondary spermatocytes, spermatids and sperm, reduce the total number of chromosomes per cell in the testis. Thirdly, the great increase in numbers of these latter cell types influence not only the concentrations by dilution of the cell population but also the absolute values by adding materials to the testis as a whole.

That the PNAP concentration was highest at ten and twenty days is entirely consistent with the cytologic picture. When, however, the cytoplasmic basophilia was examined in these two groups, it was found to be more intense than in corresponding cells in the adult group and further, that it was somewhat greater in the ten than in the twenty day testis. Thus it would seem that testes of young rats have a higher concentration of PNA than can be accounted for entirely on the basis of the cell types which are present. Since slightly elevated values for PNA have been found generally in young growing tissues and greatly increased amounts, as compared to adult tissues, have been observed in embryonic tissues (Davidson, 1950) it is not surprising to find that the PNA values in the testes of young rats, which are similar in some respects to embryonic tissues, are markedly higher than in adult testes. It is worth noting that from the tenth to the twentieth day the testis undergoes a fivefold increase in weight and from the twentieth to the thirtieth day a threefold increase. Therefore, the high concentration can be attributed in part at least to the accelerated rate of growth in young testes.

At thirty and forty days the lower PNA concentrations are entirely

consistent with the cell types which are present in the tubules at those ages as well as with the cytochemical picture, the latter being similar for each of the various cell types, to that of the normal adult. Further decreases seen in the sixty-day testis would seem to be accounted for on the basis of a dilution of the total cell population by cells with high nuclear-cytoplasmic ratios.

The same comments may be applied to the ASP fraction since it roughly parallels the PNAP fraction.

The DNAP concentrations are readily correlated with the presence or absence of haploid nuclei in a given age group. Thus at ten and twenty days the DNAP concentration is high; in fact, it is a little more than twice the normal adult value. At thirty days when the first cells with haploid nuclei appear a reduction in the DNAP concentration occurs. A further reduction is seen at forty days when sperm have made their appearance. All subsequent reductions appear to be due to increases in the total numbers of cells with haploid nuclei.

The LP fraction is unique since its concentration was relatively constant at all ages which have been studied. The most readily available explanation of this observation would hold that all of the cell types in the testis contain essentially the same amount of phospholipid and thus the appearance of new cell types and increases in the number of cells have little or no effect on the concentration. According to Sinclair (1940, 1941) the phospholipids of the testis are inert. If this is true it is perhaps to be expected that the concentration of lipid phosphorus would remain relatively constant in the different age groups.

Increases in the absolute amounts of all the fractions offer no problem when we consider the tremendous increases in the cellular population, each type of cell containing a greater or lesser quantity of the various fractions.

SUMMARY AND CONCLUSIONS

Estimations of the pentose nucleic acid phosphorus, desoxypentose nucleic acid phosphorus, total nucleic acid phosphorus, acid soluble phosphorus and lipid phosphorus have been made on the testes of rats ranging from ten to one hundred eighty days of age. The concentrations of all of the phosphorus fractions, except lipid phosphorus which remained constant, decreased progressively with increasing age of the animals. Conversely in terms of absolute values (amount in both testes) each of the phosphorus fractions showed a progressive increase with advancing age of the animals.

Cytochemical observations with the Feulgen reaction and for cytoplasmic basophilia have been made. These procedures which are considered to be related, respectively, to desoxypentose nucleic acid and pentose nucleic acid were in agreement with the chemical determinations.

It was concluded that the amounts of the several phosphorus fractions which could be detected at the various ages depends on the types of cells and on the numbers of cells which are present at those ages except in very young testes where an additional relationship exists between the rapid rate of growth of the testis and the concentrations of pentose nucleic acid and acid soluble phosphorus.

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ORGANIC PHOSPHORUS COMPOUNDS IN THE TESTIS OF THE RAT UNDER EXPERIMENTAL CONDITIONS WHICH IMPAIR SPERMATOGENESIS[1,2]

WILLIAM B. McENERY[3] AND WARREN O. NELSON

From the Department of Anatomy, College of Medicine, State University of Iowa, Iowa City, Iowa

IN SPITE of the concerted efforts which have been made to clarify the functional position and metabolic significance of nucleic acids very little in the way of established interpretations can be offered. Caspersson (1950) and Brachet (1950) have discussed the significance of nucleic acids in the synthesis of cellular proteins, Claude (1940, 1941, 1942, and 1948) has shown that cytoplasmic pentose nucleic acid (PNA) occurs in combination with phospholipids and Hevesy (1948) and Davidson (1950) have pointed out certain relationships between PNA and the components of the acid-soluble phosphorus (ASP) fraction.

It has been reported that fasting and protein-poor diets (Davidson and Waymouth, 1944; Davidson, 1945), ischemia (Drochmans, 1947), adrenocorticotrophic hormone and high carbohydrate diet (Baker, *et al.*, 1948), and hypophysectomy (Geschwind, Li and Evans, 1950) all cause a decrease in pentose nucleic acid (PNA) but no change in desoxypentose nucleic acid (DNA) in the liver. McShan *et al.* (1950) reported a marked increase in PNA but only a slight increase in DNA in the crop glands of pigeons treated with lactogenic hormone. An increase in both PNA and DNA has been observed in the livers of pregnant rats (Campbell and Kosterlitz, 1947) and Stutz and Verzar (1947) reported that thymectomy did not alter the nucleic acid content of the rat liver. Mitchell (1942, 1943) has observed that x-irradiation of various normal and cancerous human tissues produces an accumulation of PNA in the cytoplasm, and Stowell

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[3] Present address: Department of Anatomy, School of Medicine, The Creighton University, Omaha, Nebraska.

(1945) reported that x-irradiation prevents the synthesis of DNA in cancerous tissue. The studies of Errera (1947) indicate a direct effect of x-irradiation on the chemical properties involved in the rigidity of the nucleoprotein complex *in situ*.

In a previous paper (McEnery and Nelson, 1952) we pointed out the scarcity of chemical and cytochemical observations on nucleic acids and other phosphorus containing constituents in the testis and reported such information for the testes of rats ranging in age from ten to one hundred and eighty days.

This report offers similar observations on the testes of hypophysectomized, estrogen-treated, x-irradiated and cyptorchid rats. The experimental procedures to which these rats were subjected were chosen for their well-known effects on spermatogenesis.

MATERIALS AND METHODS

Rats of the Long-Evans strain raised in this laboratory and of the Sprague-Dawley strain obtained from a commercial breeder were used in this study.

Animals were hypophysectomized[4] at weights varying from 150 to 250 gm. and were sacrificed 30 days post-operatively. All animals were examined for completeness of operation.

In the estrogen-treated animals treatment was initiated when the animals weighed approximately 150 gm. Subcutaneous injections of alpha estradiol,[5] were made in 50 and 100 gamma doses daily for 30 to 45 days.

In the x-ray series animals weighing 200 to 250 gm. were given 600 roentgen units applied in a single dose to the caudal half of the body and were autopsied 30 days later.[6]

In the cryptorchid series animals weighing 200–250 gm. at the time of operation were made cryptorchid by anchoring the testes to the anterior abdominal wall. One group was sacrificed 80 days post-operatively and the other 200 days after operation.

Chemical Determinations

Pentose nucleic acid phosphorus (PNAP), desoxypentose nucleic acid phosphorus (DNAP), total nucleic acid phosphorus (TNAP), acid soluble phosphorus (ASP) and lipid phosphorus (LP) were determined according to the method of Schmidt and Thannhauser (1945) as modified by Schneider (1946). Duplicate determinations were made on testicular tissue from each animal. All were wet weight determinations and moisture contents were checked in each group.

Cytological and Cytochemical Preparations

Portions of the testes were prepared for cytological and cytochemical observation according to the methods described in the first paper of this series (McEnery and Nelson, 1952).

[4] The hypophysectomized rats were obtained from the Hormone Assay Laboratory of Chicago, Illinois.

[5] We are indebted to Dr. Edward Henderson of the Schering Corporation for the alpha estradiol.

[6] We are indebted to the staff of the Radiation Research Laboratory of the State University of Iowa for invaluable assistance in irradiating these animals.

TABLE 1. THE MEAN CONCENTRATIONS OF THE FIVE P FRACTIONS AND THE CALCULATED MEAN CONCENTRATIONS OF PNA AND DNA FOR EACH EXPERIMENTAL GROUP EXPRESSED AS MG. PER 100 GM. OF FRESH TISSUE

Group	No. of rats	PNAP	DNAP	TNAP	ASP	LP	PNA	DNA
Hypophysectomy	23	87.14 ±1.664* 0.001†	23.45 ±0.481 0.001	109.87 ±1.865 0.001	81.73 ±1.857 0.001	34.47 ±1.010 0.20	923.68	236.84
Estrogen	21	86.01 ±1.993 0.001	23.24 ±0.576 0.001	110.53 ±1.653 0.001	95.28 ±1.230 0.001	33.44 ±0.512 0.20	911.70	234.72
X-ray	19	67.66 ±1.021 0.001	14.63 ±1.516 0.050	82.25 ±1.646 0.001	81.26 ±2.186 0.001	39.60 ±0.989 0.001	717.19	147.76
Cryptorchid (80-day)	18	66.52 ±2.054 0.010	17.32 ±2.235 0.010	84.04 ±2.015 0.001	78.37 ±2.349 0.001	54.85 ±1.747 0.001	705.11	174.93
Cryptorchid (200-day)	24	67.24 ±1.596 0.001	19.42 ±1.676 0.001	85.75 ±1.300 0.001	65.90 ±1.889 0.20*	56.55 ±0.962 0.001	712.74	196.14
Normal Adult (180-day)	27	60.32 ±0.639	12.30 ±0.215	75.00 ±1.002	65.33 ±1.023	32.72 ±1.065	639.29	124.23

* Standard error of the mean.

† p values (from students table of t, Fischer and Yates 1947) for the differences between the mean values of the different experimental groups and the mean values of the normal adult group.

RESULTS

Chemical Determinations

Table 1 presents the mean values for the concentration of phosphorus in the PNA, DNA, TNA, AS and L fractions, and the calculated amounts of PNA and DNA along with standard errors and p values of the differences between the means of the values obtained for these fractions in the animals of the various experimental groups as compared with the equivalent values in normal adult animals. The corresponding absolute values (calculated amounts in both testes) are given in Table 2, and the values for concentration and absolute amounts are represented graphically in Figures 1 and 2. It will be noted that the concentrations of the various phosphorus fractions showed varying degrees of increase in the different experimental series as compared to the corresponding values for the normal adult rat. On the other hand the absolute values for all fractions were consistently lower than those observed in the normal adult.[7]

The water content of the testes in the experimental groups did not differ more than five per cent from that observed in testes of normal adult rats.

[7] Values for individual analyses and numerous illustrations may be found in the Ph.D. dissertation of the senior author (McEnery, 1952).

TABLE 2. THE CALCULATED MEAN VALUES OF THE FIVE P FRACTIONS AND THE CALCULATED MEAN VALUES FOR PNA AND DNA FOR EACH EXPERIMENTAL GROUP EXPRESSED AS MG. IN BOTH TESTES

Group	No. of rats	Wt. of testes in gm.	PNAP	DNAP	TNAP	ASP	LP	PNA	DNA
Hypophysectomy	23	0.625	0.548 ±0.020* 0.001†	0.146 ±0.004 0.001	0.690 ±0.024 0.001	0.509 ±0.014 0.001	0.216 ±0.009 0.001	5.809	1.481
Estrogen	21	0.766	0.657 ±0.058 0.001	0.173 ±0.011 0.001	0.835 ±0.063 0.001	0.731 ±0.063 0.001	0.255 ±0.022 0.001	6.965	1.755
X-ray	19	0.694	0.469 ±0.012 0.001	0.101 ±0.010 0.001	0.570 ±0.015 0.001	0.564 ±0.019 0.001	0.276 ±0.012 0.001	4.975	1.025
Cryptorchid (80-day)	18	0.755	0.503 ±0.020 0.001	0.130 ±0.005 0.001	0.635 ±0.022 0.001	0.601 ±0.035 0.001	0.416 ±0.019 0.001	5.331	1.318
Cryptorchid (200-day)	24	0.790	0.531 ±0.016 0.001	0.154 ±0.006 0.001	0.678 ±0.019 0.001	0.523 ±0.021 0.001	0.449 ±0.016 0.001	5.632	1.556
Normal Adult (180-day)	27	3.100	1.901 ±0.042	0.387 ±0.009	2.366 ±0.052	2.053 ±0.043	1.024 ±0.029	20.155	3.913

* Standard error of the mean.

† p values (from students table of t, Fischer and Yates 1947) for the differences between the mean values of the different experimental groups and the mean values of the normal adult group.

Consequently differences seen in the phosphorus fractions were not influenced to any appreciable degree by changes in the moisture content.

Cytological and Cytochemical Observations

The picture in the normal adult testis has been described in a previous paper (McEnery and Nelson 1952).

Hypophysectomized Series. In the testis of the hypophysectomized rat ordinary histologic preparations show that the Leydig cells are atrophic and the tubules contain only Sertoli cells, spermatogonia and spermatocytes.

All of the nuclei of the intertubular elements including the Leydig cells, show a strong Feulgen reaction while the tubules (except for some sloughed spermatocytes which stain deeply) present a picture similar to that seen in the same cells of the normal testis.

While the intertubular cells contain little cytoplasm that which is present is strongly basophilic in methyl green pyronin preparations. The degree of cytoplasmic basophilia in the tubular cells, with the exception of some degenerating spermatocytes which are strongly basophilic, is com-

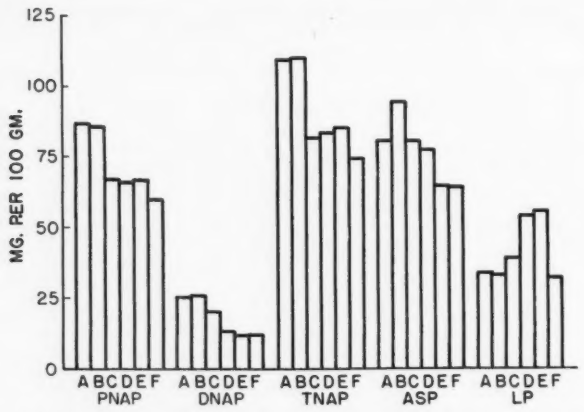

FIG. 1. Graph of the mean concentrations of the five P fractions for each experimental group and for the normal adult group.

A = Hypophysectomy C = X-ray E = Cryptorchid (20-day)
B = Estrogen-treated D = Cryptorchid (80-day) F = Normal Adult
PNAP = Pentose nucleic acid phosphorus
DNAP = Desoxypentose nucleic acid phosphorus
TNAP = Total nucleic acid phosphorus
ASP = Acid soluble phosphorus
LP = Lipid phosphorus

parable to that observed in the same cells of normal testes. In some of the degenerating cells even the nucleus is stained red and occasional red droplets occur free in the tubular lumens.

Estrogen Series. The testes of estrogen treated animals show atrophic Leydig cells and the tubules contain only Sertoli cells, spermatogonia, and spermatocytes. This picture as seen in ordinary histologic preparations is essentially the same as in hypophysectomized animals and is due to the lack of gonadotrophic hormone.

The Feulgen reaction is intense in the nuclei of all intertubular cells. The nuclei of the Sertoli cells contain little Feulgen-positive material while those of the spermatogonia and spermatocytes stain deeply.

The quantity and distribution of cytoplasmic basophilia throughout the testes of these animals appears to be essentially the same as has been described for the testes of hypophysectomized animals. There are, however, few of the degenerating spermatocytes which are so highly basophilic in the latter animals.

X-ray Series. The x-irradiated testes show intertubular areas with an apparent increase in both number and size of the Leydig cells. In x-irradiated testes an extremely variable picture is found in the tubules, some

containing only Sertoli cells while others contain sperm, occasional spermatids, a few large basal cells, and some unidentifiable cellular remnants.

The Feulgen reaction in the cell nuclei of the intertubular areas is essentially similar to that seen in the cells of these areas in normal adult testes. Within the tubules the Sertoli cells appear to contain a normal amount of Feulgen-positive material and such other cells as are present give an essentially normal reaction except for the nuclei of the basal cells and cell remnants, both of which stain deeply.

In the methyl green pyronin preparations the cytoplasm of the intertubular cells show approximately the same amount of basophilia as that of corresponding cells in the normal adult testis (a few Leydig and mast cells appear to show more). The residual tubular cells are a little more basophilic than cells of the same types in normal adult testes.

Cryptorchid Series. The testes in both of these groups are essentially similar and will be discussed together. They show the customary apparent

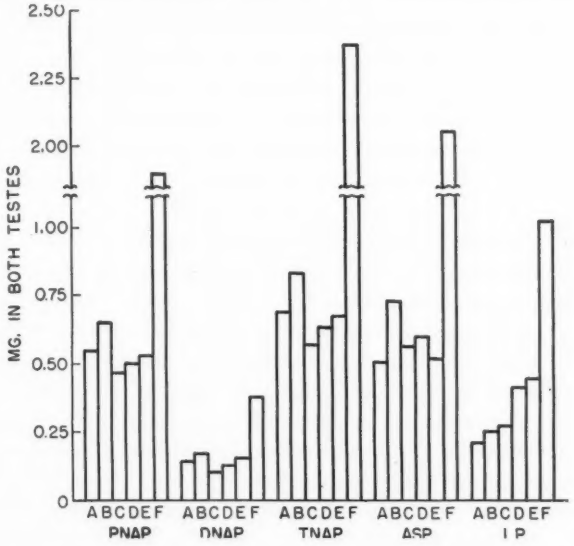

FIG. 2. Graph of the mean absolute values of the five P fractions for each experimental group and for the normal adult group.

A = Hypophysectomy C = X-ray E = Cryptorchid (200-day)
B = Estrogen-treated D = Cryptorchid (80-day) F = Normal Adult

PNAP = Pentose nucleic acid phosphorus
DNAP = Desoxypentose nucleic acid phosphorus
TNAP = Total nucleic acid phosphorus
ASP = Acid soluble phosphorus
LP = Lipid phosphorus

increase in the number and size of the Leydig cells. The tubules vary with respect to the types of cells present, some having Sertoli cells only and others, in addition, spermatogonia and even occasional spermatocytes.

With the Feulgen stain all of the nuclei of the tubular and intertubular cells show essentially the same reaction as is seen in the corresponding cells of normal adult testes.

The methyl green pyronin preparations reveal an increased amount of cytoplasmic basophilia in the Leydig cells while other intertubular cells show normal quantities. The degree of cytoplasmic basophilia in the tubular cells is also essentially normal.

DISCUSSION

In the first paper of this series (McEnery and Nelson, 1952) the significance of observations on the normal adult testis has been discussed and we have pointed out that variations in the concentration of the phosphorus fractions at various ages was chiefly due to the types of cells which occur in the seminiferous tubules at those ages. The reader is referred to that paper for a detailed discussion of the constancy of position of PNA and DNA in the cell, the relationship of those substances to the amount of cytoplasm and the presence of nuclei with diploid or haploid numbers of chromosomes, and the apparent relationship between the PNAP and ASP fractions. In the LP fractions, on the other hand, such relationships are not evident.

The PNAP concentrations in testes from hypophysectomized and estrogentrated animals showed substantial increases over those found in testes of normal adult rats. Physiologically these two conditions are similar, in that gonadotrophins are absent, and the testicular tubules contain only Sertoli cells, spermatogonia and primary spermatocytes. Furthermore, cytoplasmic basophilia in these cells is no greater than that seen in the normal adult (except for a few disintegrating cells which could not alter the picture significantly). Thus it seems likely that the increased PNAP concentration in these two groups is probably due to the presence of cells with relatively large amounts of cytoplasm rather than to any specific effect of the treatment on PNAP since the cells listed above contain larger amounts of cytoplasm than do secondary spermatocytes, spermatids and spermatozoa.

The testes of x-irradiated animals showed some increase in the concentration of PNAP. These results agree with those of Mitchell (1942, 1943) in spite of the fact that our animals were sacrificed thirty days following treatment while Mitchell examined his tissue within nine hours post-irradiation. The possibility exists that in our study we were dealing with some measure of spermatogenic repair although histologic evidence of such repair was not evident. A clear cut evaulation of the testicular picture

in terms of the content of nucleic acids is somewhat difficult in this series owing to the variable cellular content of the tubules. It can be stated, however, that the Sertoli cells were a constant feature of the tubules, that some of the tubules contained flattened basal cells and that both of these cells were significantly basophilic. Since the spermatoids and sperm contain little cytoplasm it would appear that the Sertoli cells and basal cells account for the majority of the PNA.

The increases in PNA concentration in the cryptorchid groups are not excessively large. Since the cell types which are present in the tubules contain considerable cytoplasm and since the Leydig cells show increased basophilia one might have expected higher values. Apparently the Sertoli cells in these testes are not sufficiently rich in PNA to provide the substantial increases seen in some of the other experimental series and the Leydig cells, since they are closely grouped and relatively more numerous may give the illusion of increased basophilia.

The concentrations of DNAP in the testes from hypophysectomized and estrogen-treated animals were double those found in normal adult animals. This is easily understood when we note that only cells whose nuclei possess diploid numbers of chromosomes were present.

Considerable variation was seen in the concentrations of DNAP in the testes of x-irradiated animals. However, if we take into account the variations which were encountered in the histologic condition of these testes the values for concentration of DNAP are seen to be consistent with the histologic picture. To assess this relationship more accurately it would be necessary to study a large series of animals in which individual analyses would be correlated with the varied histologic patterns in the same testes.

In the two groups of cryptorchid animals the values for the concentrations of DNAP present a somewhat baffling problem. All of the cells contain diploid numbers of chromosomes. Thus one would expect the concentrations of DNAP to be double the normal adult value, as was the case for hypophysectomized and estrogen-treated rats. Actually the concentrations were between the values observed for hypophysectomized and estrogen-treated groups and for normal adult animals. Since the Feulgen reaction in the testes of hypophysectomized and estrogen-treated animals is similar to that seen in normal adult testes it is possible that the Sertoli cells, which appear to be the predominant type of cell in the testes of cryptorchid rats, do not have as high a content of DNA as do the spermatogonia and spermatocytes which are abundant in the testes of hypophysectomized and estrogen-treated rats. That these latter cells contain larger amounts of DNA than are present in other intratubular cells has been shown by Caspersson's (1933, 1936) studies utilizing absorption microspectrophotometric techniques.

The concentration of ASP in the testes of hypophysectomized animals

paralleled that of PNAP both being higher than the corresponding values for normal adult testes and, as noted earlier for PNAP, are probably related to the types of cells which are present. On the other hand an even higher value for ASP was seen in the estrogen-treated group. A suggestion of an effect of estrogen upon constituents of the acid soluble phosphorus fraction may be found in the observations of Dirscherl and Hauptmann (1950) who reported that estrogens stimulate anaerobic glycolysis and respiration in liver slices and of McShan, Meyer and Erway (1947) and Case and Dickens (1948) who found that some estrogens inhibit the succinoxidase and cytochrome oxidase systems.

In the x-irradiated animals the ASP concentration closely paralleled the values for PNAP and therefore the comments made for PNAP apply in this case.

The concentration of ASP in the testes of eighty-day cryptorchid rats was somewhat higher than in normal adults while in the two hundred-day cryptorchids the concentration was similar to the normal value. We are unable to offer an explanation for these variations.

The observation that the concentration of the LP fraction in the testes of hypophysectomized and estrogen-treated rats does not differ from the normal value would seem to confirm the reports of Sinclair (1940, 1941) that the testicular phospholipids are inert.

The testes of x-irradiated animals and of the two groups of cryptorchid animals showed concentrations of LP which were significantly higher than the corresponding value for normal adult testes. It is noteworthy that the Sertoli cells are a constant feature as well as the predominant type of cell in these groups and it might be offered as a possible explanation that the Sertoli cell contains a larger quantity of phospholipid. On the other hand the experimental conditions imposed in the cryptorchid and x-irradiated animals may possibly have altered directly the phospholipid content. It is obvious, however, that the present status of our knowledge regarding phospholipid metabolism renders extremely difficult the proposal of any sound interpretation of these observations.

The absolute values for all fractions in the experimental series were lower than the normal adult values. This is not surprising since the testes of all of the experimental animals were much smaller than normal and had various types of cells missing from their seminiferous tubules.

SUMMARY AND CONCLUSIONS

The testes of hypophysectomized, estrogen-treated, x-irradiated and cryptorchid rats were analyzed for pentose nucleic acid phosphorus, desoxypentose nucleic acid phosphorus, total nucleic acid phosphorus, acid soluble phosphorus and lipid phosphorus. Observations also were made

on routine histologic preparations and on cytochemical preparations with the Feulgen reaction and for cytoplasmic basophilia. The latter two procedures are considered to be related to desoxypentose nucleic acid and pentose nucleic acid, respectively.

In testes from hypophysectomized and estrogen-treated animals the concentrations of all phosphorus fractions, except the lipid fraction, showed definite increases over the normal adult values.

In testes from x-irradiated animals moderate increases in the concentrations of all phosphorus fractions were seen.

In the cryptorchid rats a striking increase in the concentrations of the lipid fraction and a definite increase in the concentration of desoxypentose nucleic acid phosphorus were observed. The other fractions in testes from these animals showed only moderate increases in concentration.

The absolute values for all fractions in each of the experimental series were considerably lower than the equivalent values for normal adult animals. This is in keeping with the reduced size of the testes.

The concentrations of the various phosphorus fractions in the testes of hypophysectomized and estrogen-treated animals showed rather definite patterns which appeared to depend upon the types of cells which are present in such testes. On the other hand the values for the concentration of the several phosphorus fractions in testes of x-irradiated and cryptorchid animals present certain inconsistencies which may be due to physical and/or chemical alterations which are superimposed on such changes as may have occurred as a result of alterations in the cell populations. Further evaluation of these factors and their influences will require additional and more detailed studies.

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NOTES AND COMMENTS

SERUM ANTIDIURETIC SUBSTANCE (ADS) IN THE SHEEP AND COW

Although serum ADS has been found in rats (Birnie *et al.*, 1949); in humans (Lloyd and Lobotsky, 1950; Hawker, 1952a and 1952b); and in the kangaroo rat (Ames and van Dyke, 1952) a quantitative estimation of it in the sera of sheep and cows has not previously been reported.

This note reports the finding of serum ADS in sheep and cattle.

PROCEDURE

The method of Birnie *et al.* (1949)for measuring ADS in rats' sera was used in these experiments; the ADS units have been quantitated in terms of milliunits of pitressin (Parke Davis pitressin 20 U. per ml.). Serum ADS was assayed in the following groups:

A. Sheep
- (1) Non-pregnant, non-lactating ewes (8 animals)
- (2) Pregnant, non-lactating ewes (4 animals)

B. Cattle (Dairy and Beef)
- (1) Non-pregnant, non-lactating dairy cows (8 animals)
- (2) Non-pregnant, lactating dairy cows (8 animals)
- (3) Pregnant, non-lactating dairy cows (8 animals)
- (4) Pregnant, lactating dairy cows (6 animals)
- (5) Dairy bull (1 animal)
- (6) Non-pregnant, non-lactating beef cows (3 animals)
- (7) Beef bull (1 animal)

The dairy cows were graded into Grades I, II and III, according to their capacity to produce milk. The Grade I cows belonged to a record-holding herd for milk production in Queensland, Australia.

TABLE 1. ADS CONCENTRATION IN SHEEP DURING NON-PREGNANCY AND PREGNANCY

Species	Type	No. assayed	No. of assays	mμ ADH/ml. serum	mμ ADH/ml. (mean)
Sheep	non-pregnant & non-lactating	8	8	0.00 0.50 0.35 0.75 0.30 2.00 2.10 1.25	1.16
Sheep	pregnant & non-lactating	4	4	4.60 5.85 6.10 3.85	5.10

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RESULTS

A. Serum ADS in Sheep

Serum ADS was found in both the non-pregnant and pregnant sheep, with a higher concentration occurring during pregnancy, as shown in Table 1.

B. Serum ADS in Cattle

An antidiuretic substance was also determined in the sera of the dairy and beef cattle examined, and the results are presented in Table 2.

TABLE 2. ADS CONCENTRATION IN DAIRY AND BEEF CATTLE DURING NON-PREGNANCY, PREGNANCY AND LACTATION

Cattle	Type	No. assayed	Grade	mμ ADH/ml. serum	Mean	Reproductive state
Dairy	non-pregnant & non-lactating	2	1	4.00 3.20	3.60	nulliparous pre-puberal
	non-pregnant & non-lactating	3	1	5.40 5.65 5.05	5.37	nilliparous post-puberal
	non-pregnant & lactating	4	1	7.70 5.80 7.10 8.00	7.15	parous
	pregnant & non-lactating	4	1	5.90 7.15 7.50 7.55	7.02	—
	non-pregnant & non-lactating	4	2	6.00 5.05 7.35 7.70	6.52	parous
	non-pregnant & lactating	4	3	4.95 3.85 5.80 3.30	4.47	parous
	pregnant & non-lactating	4	3	6.20 4.15 5.00 5.15	5.12	—
	pregnant & lactating	6	1	7.25 4.35 3.20 5.95 5.75 5.30	5.30	—
	Bull	1	1		3.45	—
Beef	non-pregnant & non-lactating	2		4.30 3.40	3.85	nulliparous post-puberal
	Bull	1			2.90	—

Reference to Table 2 shows that in any type examined the better the dairy cow is graded for milk production, the higher is her serum ADS level.

SUMMARY AND CONCLUSIONS

(1) A substance possessing antidiuretic properties (ADS) is present in the freshly drawn sera of sheep and cattle.

(2) In ewes the serum ADS level is higher during pregnancy than in the non-pregnant state.

(3) In dairy cows of Grade I milking strain the serum ADS level is lowest in prepuberal heifers, and shows increments with sexual maturity, pregnancy and lactation.

Acknowledgements

I am grateful to the National Health and Medical Research Council (Australia) for their grant which enabled this work to be undertaken.

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ROSS W. HAWKER

Department of Physiology
The University of Queensland
Brisbane, Australia

SERUM ANTIDIURETIC SUBSTANCE (ADS) IN THE HUMAN FEMALE

A substance possessing antidiuretic properties (and henceforth referred to here as ADS) was found in the serum of rats by Birnie *et al.* (1949); in the human by Lloyd and Lobotsky (1950), and by Hawker (1952a and 1952b); and in the kangaroo rat, Dipodomys merriami, by Ames and van Dyke (1952). Birnie *et al.* (1949) consider that ADS found in rat serum is probably identical with the antidiuretic hormone (ADH) produced by the posterior pituitary gland.

This paper reports the finding of ADS in the adult human female during the normal menstrual cycle, pregnancy and the immediate post partum period.

PROCEDURE

Serum ADS was assayed according to the method of Birnie *et al.*, (1949). A graph was constructed correlating ADS units with milliunit concentrations of freshly prepared

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pitressin (Parke Davis. 20 U. per ml.). Serum ADS was assayed in the following groups:

(1) Healthy non-pregnant females—mostly (medical) students—aged between 17–22 years; in all, 65 assays were done on 28 individuals at different times during the menstrual cycle.

(2) Healthy pregnant women—usually from the 10th or 12th week following the last menstrual period; a total of 61 assays were performed on 24 women.

(3) Healthy women in immediate post partum period; 50 assays were done on 11 women in the first 4–5 days after delivery.

RESULTS

This work shows that ADS is present in the serum of the healthy, cyclically menstruating female. Figure 1 shows clearly that during the menstrual cycle the serum ADS level is most commonly 0–1 mμ ADH/ml. of serum; the arithmetic mean of the 65 assays examined was 1.43 (S.D. ±1.18) mμ ADH/ml. of serum. Figure 1 also reveals that during pregnancy the serum ADS level is higher than during the menstrual cycle, and the distribution is wider. Here the serum ADS level is most commonly 5–6 mμ ADH/ml. of serum; the mean of the 61 assays was 4.53 mμ (S.D. ±1.34 ADH/ml. of serum.

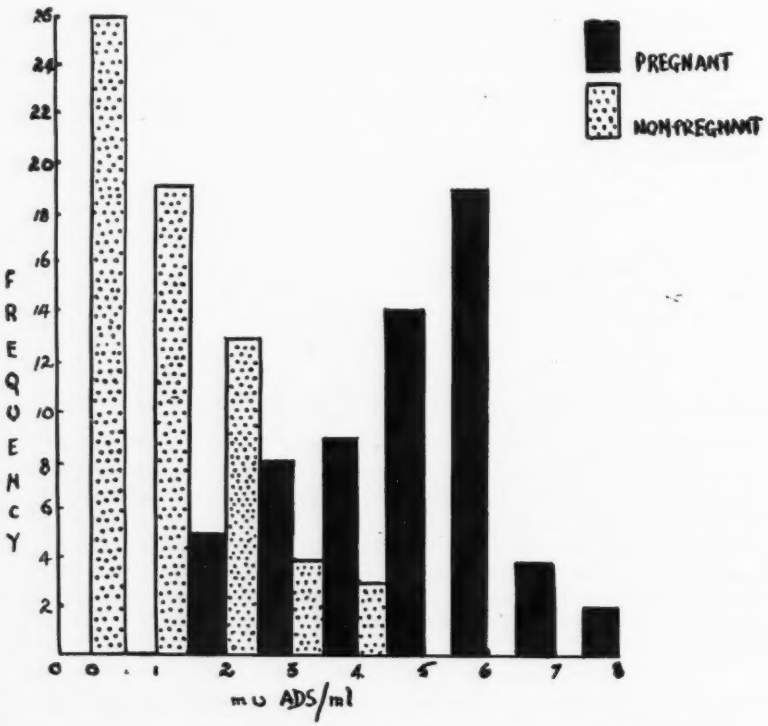

FIG. I. Histogram of serum ADS levels in pregnant and non-pregnant women.

The serum ADS levels in the weeks of the menstrual cycle are expressed in Table 1.

TABLE 1. ADS CONCENTRATION DURING THE MENSTRUAL CYCLE

		Milliunits of ADH/ml. serum	
Menstrual Cycle	Whole Cycle	1.43 (S.D. ±1.18)	Mean of 65 assays
	0– 7 days	1.48	Mean of 17 assays
	8–14 days	0.95	Mean of 10 assays
	15–21 days	1.72	Mean of 12 assays
	22–28 days	1.45	Mean of 15 assays

Some results are not included, as a record of the time in the menstrual cycle, when the assays were done, was not obtained.

The serum ADS levels during pregnancy are presented in Table 2, from which it will be seen that the level appears to remain comparatively steady throughout pregnancy with perhaps a slight over-all upward trend as pregnancy advances.

TABLE 2. ADS CONCENTRATION DURING NORMAL PREGNANCY AND FIRST DAY POST PARTUM

Time	Milliunits ADH/ml. serum (mean)	
1st Trimester	3.69	(4 assays)
2nd Trimester	4.21	(15 assays)
3rd Trimester	4.37	(21 assays)
Last month of pregnancy	4.37	(9 assays)
1st day post partum	5.50	(11 assays)

Some results are not included in the table because a record of the period of gestation was not obtained.

Immediately after parturition the serum ADS levels are higher than are found in non-pregnant women, and reference to Table 3 will show that a correlation appears to exist between the level of serum ADS and the onset of lactation. Here there occurred an initial post partum fall in the serum ADS level, and lactation began within a few hours after the serum ADS approached or reached its lowest recorded initial level. In no instance did lactation begin before the *initial* fall in the serum ADS level.

TABLE 3. ADS CONCENTRATION AND ONSET OF LACTATION

Case	Days after parturition				
	Milliunits ADH/ml. serum				
	1	2	3	4	5
1	7.0	5.85	5.0	*4.15*	5.2
2	4.9	4.45	*4.0*		
3	3.45	4.85	*2.35*	6.15	
4	*2.5*	3.15	3.35	5.1	
5	5.95	5.2	*2.9*	3.0	5.1
6	4.45	4.3	*3.35*	7.1	2.3
7	4.4	3.84	*0*	7.2	3.9
8	7.85	*3.3*	5.3		
9	5.5	*5.15*	7.2	2.95	4.1
10	5.6	5.6	5.85	*3.45*	3.9
11	5.9	*3.1*	4.9	2.75	5.45

Italicized figures = Lactation commenced within hours of this point.

The mean concentrations of ADH/ml. of serum for the eleven cases on the first day post partum and at the onset of lactation were 5.50 (S.D. ±1.29) and 3.11 (S.D. ±1.39) respectively. (First post partum reading for Case No. 4 excluded as lactation commenced on the first day post partum.)

SUMMARY AND CONCLUSIONS

(i) A substance possessing antidiuretic properties (ADS) is present in the freshly drawn serum of women during the normal menstrual cycle, normal pregnancy and early post partum period.

(ii) The serum ADS level is higher during pregnancy than during the menstrual cycle.

(iii) It is postulated that this high level during pregnancy accounts for some of the water retention of pregnancy.

(iv) A correlation appears to exist between the level of serum ADS after parturition and the onset of lactation. In each of the eleven cases examined, lactation always began after an initial fall in the serum ADS level.

Acknowledgements

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I wish to thank Professor W. V. Macfarlane of the Department of Physiology and Professor, H. J. G. Hines of the Department of Biochemistry, University of Queensland, for their many helpful suggestions and criticisms during the course of this study.

I am also indebted to Dr. R. Salter and Dr. K. G. Cockburn of the Brisbane Women's Hospital for their co-operation and I am pleased to acknowledge the technical assistance given to me during the course of these studies by Miss Joan Hunter, Department of Physiology, University of Queensland.

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ROSS W. HAWKER

The University of Queensland
Department of Physiology
Brisbane, Australia

THE 1953 ANNUAL MEETING

The Thirty-fifth Annual Meeting of The Endocrine Society will be held in the Hotel Statler, New York, New York, Thursday, Friday, and Saturday, May 28–29–30, 1953.

The Committee on Local Arrangements is Dr. Earl T. Engle, Chairman, with Drs. Rulon W. Rawson and Sidney C. Werner as members of the Committee.

All Scientific Sessions will be held in the Hotel Statler. The rooms in which each session will be held will be announced in the program and on the hotel bulletin board. The Annual Dinner is scheduled for Friday, May 29, at 7:30 P.M., preceded by cocktails at 6:30 P.M.

All members are urged to make hotel reservations immediately inasmuch as the hotels expect to be filled to capacity. Reservations should be made directly with the Hotel Statler, advising time of arrival and departure date. Make your reservations now and avoid disappointment.

1953 AWARDS AND FELLOWSHIPS

Awards and Fellowships presented by The Endocrine Society carry no obligation by the recipient to the Society or to the donors.

FELLOWSHIPS

Society Fellowships are designed to assist men or women of exceptional promise in furthering their educational training and advancement toward a scientific career in endocrinology. Fellowships may be awarded to an individual who possesses the Ph.D. or M.D. degree or to a candidate for either of these degrees. The stipend, which will not exceed $5,000, may be divided into two Fellowships in varying amounts in accordance with the qualifications of the appointee. The Committee will, in reviewing the proposed program of study, consider the amount of time which the Fellow intends to spend in course work and/or teaching. He must present evidence of scientific ability as attested by studies completed or in progress and/or the recommendation of responsible individuals. He must submit a program of proposed study, indicate one or more institutions where the proposed program will be followed, and submit statements of approval from the investigators with whom he proposes to conduct his research. He must serve full time if awarded a Fellowship. A small amount of time (10 to 15 per cent) may be allotted for course work or for participation in teaching, the latter purely on a voluntary basis.

THE AYERST, McKENNA AND HARRISON FELLOWSHIP

This Fellowship will not be awarded in 1953 in order that it may accumulate toward a $5,000 Fellowship to be awarded in 1954.

THE SCHERING FELLOWSHIP IN ENDOCRINOLOGY

The Schering Fellowship in Endocrinology was established in 1949 and

the first recipient was Dr. D. Laurence Wilson in 1950. Dr. John D. Stoekle was chosen as the recipient of the 1951 Fellowship. Inasmuch as he was unable to accept due to a call to military service, the same Fellowship was reawarded in 1952 to Dr. C. L. Courtright. The 1953 Schering Fellowship will be in the amount of $5,000 and will henceforth be given in the same amount on alternate years.

AWARDS

THE SQUIBB INSTITUTE FOR MEDICAL RESEARCH AWARD

This Award of $1,000 was established in 1939 by The Squibb Institute for Medical Research and was given first in 1940 to Dr. George W. Corner; in 1941 to Dr. Philip E. Smith; in 1942 to Dr. Fred C. Koch; in 1944 to Dr. E. A. Doisy; in 1945 to Dr. E. C. Kendall; in 1946 to Dr. Carl G. Hartman; in 1947 to Drs. Carl F. and Gerty T. Cori; in 1948 to Dr. Fuller Albright; in 1949 to Dr. Herbert M. Evans; in 1950 to Dr. C. N. H. Long; and in 1951 to Dr. J. B. Collip. No Award was given in 1943. This Award was increased to the amount of $2,500 beginning with the 1952 Award and the recipient was Dr. James H. Means. A special committee of five members of the Society selects the recipient from among investigators in the United States or Canada, on the basis of outstanding contributions to endocrinology.

THE CIBA AWARD

The Ciba Award, to recognize the meritorious accomplishment of an investigator not more than thirty-five years of age in the field of clinical or pre-clinical endocrinology, was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood; in 1945 to Dr. Jane A. Russell; in 1946 to Dr. Martin M. Hoffman; in 1947 to Dr. Choh Hao Li; in 1948 to Dr. Carl Heller; in 1949 to Dr. George Sayers; in 1950 to Dr. Oscar M. Hechter; and in 1951 to Dr. Albert Segaloff. Effective in 1952, the Award was increased from $1,200 to $1,800 and the recipient was Dr. Seymour Lieberman. If within twenty-four months of the date of the Award, the recipient should choose to use it toward further study in a Laboratory other than that in which he is at present working, it will be increased to $2,500.

NOMINATIONS

Each member has the privilege of making one nomination for each Fellowship or Award. A nomination should be accompanied by a statement of the importance of the nominee's contributions to, or interest in, endocrinology and by a bibliography of the nominee's most important publications, with reprints if possible. The nominations should be made on special application forms which may be obtained from the Secretary, Dr. Henry H. Turner, 1200 North Walker Street, Oklahoma City, Oklahoma, and returned to him not later than March 1, 1953.